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DOCUMENT-IDENTIFIER: US 5736139 A
TITLE: Treatment of Clostridium difficile induced disease

Abstract Text (1):
The present provides neutralizing antitoxin directed against C. difficile toxins. These antitoxins are produced in arian species using soluble recombinant C. difficile toxin proteins. The avian antitoxins are designed so as to be orally administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). Solid forms of the antitoxin may comprise an enteric coating. These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin. The invention further provides vaccines capable of protecting a vaccinated recipient from the morbidity and mortality associated with C. difficile infection. These vaccines are useful for administration to humans and other animals at risk of exposure to C. difficile toxins.

Brief Summary Text (2):
The present invention relates to clostridial antitoxin and vaccine therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of clostridial toxins are provided. Vaccines which prevent the morbidity and mortality associated with clostridial diseases are provided.

Brief Summary Text (6):
Perhaps because of their significance for human and veterinary medicine, much research has been conducted on these toxins, in particular those of C. botulinum and C. difficile.

Brief Summary Text (7):
C. botulinum

Brief Summary Text (8):
Several strains of Clostridium botulinum produce toxins of significance to human and animal health. [C. L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990).] The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death. Particularly at risk for developing clostridial diseases are neonates mad humans and animals in poor health (e.g., those suffering from diseases associated with old age or immunodeficiency diseases).

Brief Summary Text (9):
Clostridium botulinum produces the most poisonous biological toxin known. The lethal human dose is a mere 10.sup.-9 mg/kg bodyweight for toxin in the bloodstream. Botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, it results in respiratory failure that can cause death. [S. Arnon, J. Infect. Dis. 154:201-206 (1986).]

Brief Summary Text (10):
C. botulinum spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produces toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980).]

Brief Summary Text (11):
Botulism disease may be grouped into four types, based on the method of introduction of toxin into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin. There were 355 cases of food-borne botulism in the United States between 1976 and 1984. [K. L.

MacDonald et al., Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups. [C. O. Tacket et al., Am. J. Med. 76:794 (1984).] Wound-induced botulism results from C. botulinum penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream. Since 1950, thirty cases of wound botulism have been reported. [M. N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B. D. Davis et al., (eds.), Microbiology, 4th edition, J. B. Lippincott Co. (1990).] Inhalation botulism results when the toxin is inhaled. Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin. 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare [D. R. Franz et al., in Botulinum and Tetanus Neurotoxins, B. R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-476]. Infectious infant botulism results from C. botulinum colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).] There have been 500 cases reported since it was first recognized in 1976. [M. N. Swartz, supra.]

Brief Summary Text (12):
Infant botulism strikes infants who are three weeks to eleven months old (greater than 90% of the cases are infants less than six months). [S. Arnon, J. Infect. Dis. 154:201 (1986).] It is believed that infants are susceptible, due, in large part, to the absence of the full adult complement of intestinal microflora. The benign microflora present in the adult intestine provide an acidic environment that is not favorable to colonization by C. botulinum. Infants begin life with a sterile intestine which is gradually colonized by microflora. Because of the limited microflora present in early infancy, the intestinal environment is not as acidic, allowing for C. botulinum spore germination, growth, and toxin production. In this regard, some adults who have undergone antibiotic therapy which alters intestinal microflora become more susceptible to botulism.

Brief Summary Text (13):
An additional factor accounting for infant susceptibility to infectious botulism is the immaturity of the infant immune system. The mature immune system is sensitized to bacterial antigens and produces protective antibodies. Secretory IgA produced in the adult intestine has the ability to agglutinate vegetative cells of C. botulinum. [S. Arnon, J. Infect. Dis. 154:201 (1986).] Secretory IgA may also act by preventing intestinal bacteria and their products from crossing the cells of the intestine. [S. Arnon, Epidemiol. Rev. 3:45 (1981).] The infant immune system is not primed to do this.

Brief Summary Text (16):
Different strains of Clostridium botulinum each produce antigenically distinct toxin designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism; types B, E and F have also been implicated in a smaller percentage of the food botulism cases [H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, supra]. Nearly all cases of infant botulism have been caused by bacteria producing either type A or type B toxin. (Exceptionally, one New Mexico case was caused by Clostridium botulinum producing type F toxin and another by Clostridium botulinum producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

Brief Summary Text (20):
Infant botulism has been implicated as the cause of mortality in some cases of Sudden Infant Death Syndrome (SIDS, also known as crib death). SIDS is officially recognized as infant death that is sudden and unexpected and that remained unexplained despite complete post-mortem examination. The link of SIDS to infant botulism came when fecal or blood specimens taken at autopsy from SIDS infants were found to contain C. botulinum organisms and/or toxin in 3-4% of cases analyzed. [D. R. Peterson et al., Rev. Infect. Dis. 1:630 (1979).] In contrast, only 1 of 160 healthy infants (0.6%) had C. botulinum organisms in the feces and no botulinal toxin. (S. Arnon et al., Lancet, pp. 1273-76, Jun. 17, 1978.)

Brief Summary Text (23):
Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A C. botulinum vaccine comprising chemically inactivated (i.e., formaldehyde-treated) type A, B,C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages.

First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

Brief Summary Text (24):
What is needed are safe and effective vaccine preparations for administration to those at risk of exposure to C. botulinum toxins.

Brief Summary Paragraph Table (1):
TABLE 1 Clostridium Species of Medical and Veterinary Importance* Species Disease C. aminovalericum Bacteriuria (pregnant women) C. argentinense Infected wounds; Bacteremia; Botulism; Infections of amniotic fluid C. baratii Infected war wounds; Peritonitis; Infectious processes of the eye, ear and prostate C. beijerinckikii Infected wounds C. bifermentans Infected wounds; Abscesses; Gas Gangrene; Bacteremia C. botulinum Food poisoning; Botulism (wound, food, infant) C. butyricum Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses; Bacteremia C. cadaveris Abscesses; Infected wounds C. carnis Soft tissue infections; Bacteremia C. chauvoei Blackleg C. clostridioforme Abdominal, cervical, scrotal, pleural, and other infections; Septicemia; Peritonitis; Appendicitis C. cochlearium Isolated from human disease processes, but role in disease unknown. C. difficile Antimicrobial-associated diarrhea; Pseudo- membranous enterocolitis; Bacteremia; Pyogenic infections C. fallax Soft tissue infections C. ghnoii Soft tissue infections C. glycolicum Wound infections; Abscesses; Peritonitis C. hastiforme Infected war wounds; Bacteremia; Abscesses C. histolyticum Infected war wounds; Gas gangrene; Gingival plaque isolate C. indolis Gastrointestinal tract infections C. innocuum Gastrointestinal tract infections; Empyema C. irregulare Penile lesions C. leptum Isolated from human disease processes, but role in disease unknown. C. limosum Bacteremia; Peritonitis; Pulmonary infections C. malenominatum Various infectious processes C. novyi Infected wounds; Gas gangrene; Blackleg, Big head (ovine); Redwater disease (bovine) C. oroticum Urinary tract infections; Rectal abscesses C. paraputrificum Bacteremia; Peritonitis; Infected wounds; Appendicitis C. perfringens Gas gangrene; Anaerobic cellulitis; Intra- abdominal abscesses; Soft tissue infections; Food poisoning; Necrotizing pneumonia; Empyema; Meningitis; Bacteremia; Uterine Infections; Enteritis necrotans; Lamb dysentery; Struck; Ovine Enterotoxemia; C. putrefaciens Bacteriuria (Pregnant women with bacteremia) C. putrificum Abscesses; Infected wounds; Bacteremia C. ramosum Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia C. sartagoforme Isolated from human disease processes, but role in disease unknown. C. septicum Gas gangrene; Bacteremia; Suppurative infections; Necrotizing enterocolitis; Braxy C. sordellii Gas gangrene; Wound infections; Penile lesions; Bacteremia; Abscesses; Abdominal and vaginal infections C. sphenoides Appendicitis; Bacteremia; Bone and soft tissue infections; Intraperitoneal infections; Infected war wounds; Visceral gas gangrene; Renal abscesses C. sporogenes Gas gangrene; Bacteremia; Endocarditis; central nervous system and pleuropulmonary infections; Penile lesions; Infected war wounds; Other pyogenic infections C. subterminale Bacteremia; Empyema; Biliary tract, soft tissue and bone infections C. symbiosum Liver abscesses; Bacteremia; Infections resulting due to bowel flora C. tertium Gas gangrene; Appendicitis; Brain abscesses; Intestinal tract and soft tissue infections; Infected war wounds; Periodontitis; Bacteremia C. tetani Tetanus; Infected gums and teeth; Corneal ulcerations; Mastoid and middle ear infections; Intraperitoneal infections; Tetanus neonatorum; Postpartum uterine infections; Soft tissue infections, especially related to trauma (including abrasions and lacerations); Infections related to use of contaminated needles C. thermosaccharolyticum Isolated from human disease processes, but role in disease unknown.

*Compiled from P. G. Engelkirk et al. "Classification", Principles and Practice of Clinical Anaerobic Bacteriology, pp. 22-23, Star Publishing Co., Belmont, CA (1992); J. Stephen and R. A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in Bacterial Toxins, 2d ed., pp. 66-67, American Society for Microbiology (1986); R. Berkow and A. J. Fletcher (eds.), "Bacterial Diseases," Merck Manual of Diagnosis and Therapy, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O. H. Sigmund and C. M. Fraser (eds.), "Clostridial Infections," Merck Veterinary Manual, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

Drawing Description Text (2):
FIG. 1 shows the reactivity of anti-C. botulinum IgY by Western blot.

Drawing Description Text (3):
FIG. 2 shows the IgY antibody titer to C. botulinum type A toxoid in eggs, measured by ELISA.

Drawing Description Text (22):
FIG. 21 is an SDS-PAGE gel showing the purification of recombinant C. difficile toxin B fusion protein.

Drawing Description Text (26):
FIG. 25 shows C. botulinum type A toxin expression constructs; constructs used to provide C. botulinum or C. difficile sequences are also shown.

Drawing Description Text (27):
FIG. 26 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of recombinant C. botulinum type A toxin fusion proteins.

Drawing Description Text (28):
FIG. 27 shows C. botulinum type A toxin expression constructs; constructs used to provide C. botulinum sequences are also shown. Polyhistidine tags are represented by "HHHHH" (SEQ ID NO:32).

Drawing Description Text (34):
FIG. 33 shows an SDS-PAGE gel stained with Coomaisse blue and a Western blot showing the expression of the several recombinant C. difficile toxin A fusion proteins in E. coli host cells.

Drawing Description Text (35):
FIG. 34 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of recombinant C. difficile toxin A and B fusion proteins.

Drawing Description Text (51):
FIG. 50 shows a Western blot showing C. difficile toxin A levels in culture supernatant, column flow through and column eluate from an affinity purification column.

Drawing Description Text (52):
FIG. 51 shows a Western blot showing C. difficile toxin A levels in culture supernatant, column flow through and column eluate from an affinity purification column.

Drawing Description Text (55):
FIG. 54 is an native PAGE gel stained with Coomaisse blue and a Western blot showing C. difficile toxin B levels in a commercial toxin B preparation and column flow through and column eluate from an affinity purification column.

Detailed Description Text (5):
As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., C. difficile toxin A or B and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the C. difficile protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

Detailed Description Text (6):
As used herein the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

Detailed Description Text (7):
The term "protein of interest" as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow

for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

Detailed Description Text (8):
As used herein, the term "maltose binding protein" refers to the maltose binding protein of E. coli. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

Detailed Description Text (9):
As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus or both termini of a protein of interest or a fusion partner. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

Detailed Description Text (10):
The term "thioredoxin protein" when used in reference to a fusion protein refers to a the thioredoxin protein of E. coli. It is noted that the invention is not limited by the source of the thioredoxin protein, while the E. coli thioredoxin protein is particularly preferred, thioredoxin proteins may be obtained from several sources. A portion of the thioredoxin protein may be added to a protein of interest to generate a fusion protein; a portion of the thioredoxin protein may enhance the solubility of the resulting fusion protein when expressed in a bacterial host.

Detailed Description Text (11):
As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. The purification of antitoxin may be accomplished by a variety of means including the extraction and precipitation of avian antitoxin from eggs using polyethylene glycol. Purification of anticlostridal antitoxin may also be accomplished by affinity chromatography on a resin comprising a portion of a clostridial toxin protein. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample. Additionally, the recombinant toxin polypeptides are purified by the removal of host cell components such as lipopolysaccharide (e.g., endotoxin).

Detailed Description Text (21):
The term "therapeutic vaccine" when used in reference to a vaccine comprising one or more recombinant clostridial toxin fusion proteins means that the vaccine contains an immunologically-effective amount of the fusion proteins (i.e., the immunogens.

Detailed Description Text (27):
The term "monovalent" when used in reference to a clostridial vaccine refers to a vaccine which is capable of provoking an immune response in a host (i.e., a subject) animal directed against a single type of clostridial toxin. For example, if immunization of a host with C. difficile type A toxin vaccine induces antibodies in the immunized host which protect against a challenge with type A toxin but not against challenge with type B toxin, then the type A vaccine is said to be monovalent. In contrast, a "multivalent" vaccine provokes an immune response in a host animal directed against several (i.e., more than one) clostridial toxins. For example, if immunization of a host with a vaccine comprising C. difficile type A and B toxins induces the production of antibodies which protect the host against a challenge with both type A and B toxin, the vaccine is said to be multivalent (in particular, this hypothetical vaccine is bivalent).

Detailed Description Text (29):
The term "subject" when used in reference to administration of compositions comprising antitoxins or vaccines refers to the recipient animal to whom said antitoxins or vaccines are administered. The subject may be any animal, including mammals and more

particularly, humans, in which it is desirable to administer said compositions. The subject may have been previously exposed to one or more C. difficile toxins prior to administration of said compositions (this constitutes therapeutic administration to the subject). Alternatively, the subject may not have been previously exposed to C. difficile toxins prior to administration of said compositions (this constitutes prophylactic administration to the subject).

Detailed Description Text (37):
The term "toxin" when used in reference to toxins produced by members (i.e., species and strains) of the genus Clostridium refers to the proteins which are poisonous to tissue(s). For example, the toxins produced by C. difficile are poisonous to intestinal tissues; the toxins produced by C. botulinum are poisonous to nerve tissue.

Detailed Description Text (43):
The term "parenteral administration" refers to the delivery of a composition, such as a composition comprising an antitoxin or vaccine, by a route other than through the gastrointestinal tract (e.g., oral delivery) or the lungs. In particular, parenteral administration may be via intravenous, subcutaneous, intramuscular or intramedullary (i.e., intrathecal) injection.

Detailed Description Text (52):
The present invention provides compositions comprising an avian neutralizing antitoxin directed against a portion of C. difficile toxin A and a portion of C. difficile toxin B. The antitoxins find use in humans and other animals exposed to or at risk of exposure to C. difficile. In one embodiment, the component of the avian neutralizing antitoxin directed against a portion C. difficile toxin A is directed against a first fusion protein comprising a portion of C. difficile toxin A and a second fusion protein comprising a portion of C. difficile toxin B. In yet another embodiment, both first and second fusion proteins further comprise at least one non-toxin protein sequence. In a still further embodiment, the antitoxin is directed against a portion of C. difficile toxin A comprising a portion of SEQ ID NO:6. In another embodiment, the antitoxin is directed against a portion of C. difficile toxin A, wherein the portion of SEQ ID NO:6 comprises a sequence selected from the group comprising SEQ ID NOS:7, 8 and 29. In yet another embodiment, the first and second fusion proteins comprise at least one non-toxin protein sequence. It is not intended that the present invention be limited by the nature of the non-toxin protein sequence. In one embodiment, the non-toxin protein sequence comprises a poly-histidine tract. In yet another embodiment, the non-toxin protein sequence comprises the maltose binding protein. In yet another embodiment, the non-toxin protein sequence comprises a thioredoxin protein. In a still further embodiment, the antitoxin is directed against a portion of C. difficile toxin B comprising a portion of SEQ ID NO:10. In another embodiment, the antitoxin is directed against a portion of C. difficile toxin B, wherein the portion of SEQ ID NO:10 comprises a sequence selected from the group comprising SEQ ID NOS:20, 21 and 30. In still another embodiment, the compositions comprising the avian antitoxins further comprise an enteric coating.

Detailed Description Text (58):
The invention further contemplates a method of vaccinating a subject to produce neutralizing antitoxin directed against C. difficile toxin comprising: a) providing in any order: i) a subject, ii) a first purified soluble and substantially endotoxin-free protein comprising a portion of Clostridium difficile toxin A sequence SEQ ID NO:6, and iii) a second purified soluble and substantially endotoxin-free protein comprising a portion of Clostridium difficile toxin B sequence SEQ ID NO:10; B) mixing the first and second proteins to create a therapeutic vaccine; and c) vaccinating the subject with the therapeutic vaccine so as to generate neutralizing antitoxin. The method of vaccination is not limited by the nature or species of the subject. In one embodiment the subject is a bird. In another embodiment the subject is a mammal. In yet another embodiment the subject is a human. In a still further embodiment, the method of vaccination the first and second toxin proteins further comprise at least one non-toxin protein sequence. The invention is not limited by the nature of the non-toxin protein sequence. In one embodiment, the non-toxin protein sequence comprises a poly-histidine tract. In another embodiment, the non-toxin protein sequence comprises the maltose binding protein. In yet another embodiment, the non-toxin protein sequence comprises a thioredoxin protein.

Detailed Description Text (60):
The invention further provides a fusion protein comprising at least one non-toxin protein sequence and a portion of the Clostridium difficile toxin A sequence consisting of SEQ ID NO:29. In one embodiment, the non-toxin protein sequence comprises a

thioredoxin protein. In yet another embodiment, the non-toxin protein sequence further comprises a poly-histidine tract.

Detailed Description Text (65):
The present invention contemplates vaccinating humans and other animals polypeptides derived from C. botulinum neurotoxin which are substantially endotoxin-free. These botulinal peptides are also useful for the production of antitoxin. Anti-botulinal toxin antitoxin is useful for the treatment of patients effected by or at risk of symptoms due to the action of C. botulinum toxins. The organisms, toxins and individual steps of the present invention are described separately below.

Detailed Description Text (68):
toxins from all Clostridium species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of C. butyricum, C. sordellii toxins HT and LT, toxins A, B, C, D, E, F, and G of C. botulinum and the numerous C. perfringens toxins. In one preferred embodiment, toxins A and B of C. difficile are contemplated as immunogens. Table 2 above lists various Clostridium species, their toxins and some antigens associated with disease.

Detailed Description Text (72):
In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxin from all clostridial bacteria sources (see Table 2) are contemplated as immunogens. Examples of these toxins are C. butyricum neuraminidase toxin, toxins A, B, C, D, E, F, and G from C. botulinum, C. perfringens toxins .alpha., .beta., .epsilon., and .iota., C. sordellii toxins HT and LT. In a preferred embodiment, C. difficile toxins A and B are contemplated as immunogens.

Detailed Description Text (115):
V. Vaccines Against Clostridial Species

Detailed Description Text (116):
The invention contemplates the generation of mono- and multivalent vaccines for the protection of an animal (particularly humans) against several clostridial species. Of particular interest are vaccines which stimulate the production of a humoral immune response to C. difficile, C. tetani and C. botulinum in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the clostridial species listed above. When toxin proteins are used as immunogens they are generally modified to reduce the toxicity. This modification may be by chemical or genetic (i.e., recombinant DNA technology) means. In general genetic detoxification (i.e., the expression of nontoxic fragments in a host cell) is preferred as the expression of nontoxic fragments in a host cell precludes the presence of intact, active toxin in the final preparation. However, when chemical modification is desired, the preferred toxin modification is formaldehyde treatment.

Detailed Description Text (117):
The invention contemplates that recombinant C. difficile toxin proteins be used as antigens in mono- and multivalent vaccine preparations. Soluble, substantially endotoxin-free recombinant C. difficile toxin A and or toxin B proteins may be used alone or in conjunction with either recombinant or native toxins or toxoids from C. botulinum, C. difficile and C. tetani as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of C. botulinum and C. tetani toxin proteins, a vaccine comprising C. difficile and botulinum toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against C. botulinum, C. tetani and C. difficile.

Detailed Description Text (119):
Vaccines which confer immunity against one or more of the toxin types A and B would be useful as a means of protecting animals, including humans, from the deleterious effects of C. difficile toxins. A subject may be immunized with compositions comprising one or more C. difficile toxin proteins to generate neutralizing antibodies in the subject. A subject may be immunized with a first immunogen comprising C. difficile toxin A proteins followed by a separate immunization with a second immunogen comprising C. difficile B toxin proteins to produce neutralizing antibodies directed against C. difficile toxins A and B. Alternatively, the subject may be immunized with a single immunogen comprising C. difficile toxin A and B proteins.

Detailed Description Text (120):

In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide is not optimal for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants. Another major limitation of using botulinal toxoids for the generation of antitoxins or vaccines is the high production expense. For the above reasons, the development of methods for the production of nontoxic but immunogenic C. difficile toxin proteins is desirable.

Detailed Description Text (121):
Recombinant C. difficile toxin proteins have be produced in a host cell such as E. coli in either a soluble or insoluble form. Insoluble recombinant proteins are found in inclusion bodies. Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (i.e., greater than or equal to about 0.75% of total cellular protein) in E. coli or other host cells. This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (i.e., substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in E. coli is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

Detailed Description Text (122):
The subject invention provides soluble C. difficile toxin proteins produced in economical host cells (e.g., E. coli). Further, methods for the isolation of purified soluble C. difficile toxin proteins which are suitable for immunization of humans and other animals are provided. These soluble, purified preparations of C. difficile toxin proteins provide the basis for improved vaccine preparations and facilitate the production of antitoxin.

Detailed Description Text (123):
When recombinant clostridial toxin proteins produced in gram-negative bacteria (e.g., E. coli) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenically-effective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjutants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GMDP; C.C. Biotech Corp.), RIBI adjuvant (MPL; RIBI Immunochemical Research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjutants are particularly preferred when vaccines are to be administered to humans. The route of immunization may be nasal, oral, intramuscular, intraperitoneal or subcutaneous.

Detailed Description Text (124):
The invention contemplates the use of soluble, substantially endotoxin-free preparations of fusion proteins comprising portions of C. difficile toxins A and B as vaccines. In one embodiment, the vaccine comprises a portion of a C. difficile toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising a portion of a C. difficile toxin protein and a poly-histidine tract is expressed using the pET series of expression vectors (Novagen). The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (i.e., a DE3 host strain). The production of C. difficile toxin fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express the C. difficile protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein).

Detailed Description Text (129):
The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin A and toxin B proteins, antibodies raised

against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

Detailed Description Text (130):
The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

Detailed Description Text (171):
Production of C. botulinum Type A Antitoxin in Hens

Detailed Description Text (172):
In order to determine whether antibodies could be raised against the toxin produced by clostridial pathogens, which would be effective in treating clostridial diseases, antitoxin to C. botulinum type A toxin was produced. This example involves: (a) toxin modification; (b) immunization; (c) antitoxin collection; (d) antigenicity assessment; and (e) assay of antitoxin titer.

Detailed Description Text (174):
C. botulinum type A toxoid was obtained from B. R. DasGupta. From this, the active type A neurotoxin (M.W. approximately 150 kD) was purified to greater than 99% purity, according to published methods. [B. R. DasGupta & V. Sathyamoorthy, Toxicon, 22:415 (1984).] The neurotoxin was detoxified with formaldehyde according to published methods. [B. R. Singh & B. R. DasGupta, Toxicon, 27:403 (1989).]

Detailed Description Text (176):
C. botulinum toxoid for immunization was dissolved in PBS (1 mg/ml) and was emulsified with an approximately equal volume of CFA (GIBCO) for initial immunization or IFA for booster immunization. On day zero, two white leghorn hens, obtained from local breeders, were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml inactivated toxoid emulsified in 1 ml CFA. Subsequent booster immunizations were made according to the following schedule for day of injection and toxoid amount: days 14 and 21--0.5 mg; day 171--0.75 mg; days 394, 401, 409--0.25 mg. One hen received an additional booster of 0.150 mg on day 544.

Detailed Description Text (180):
Eggs were collected from day 409 through day 423 to assess whether the toxoid was sufficiently immunogenic to raise antibody. Eggs from the two hens were pooled and antibody was collected as described in the standard PEG protocol. [Example 1(c).] Antigenicity of the botulinal toxin was assessed on Western blots. The 150 kD detoxified type A neurotoxin and unmodified, toxic, 300 kD botulinal type A complex (toxin used for intragastric route administration for animal gut neutralization experiments; see Example 6) were separated on a SDS-polyacrylamide reducing gel. The Western blot technique was performed according to the method of Towbin. [H. Towbin et at., Proc. Natl. Acad. Sci. USA, 76:4350 (1979).] Ten .mu.g samples of C. botulinum complex and toxoid were dissolved in SDS reducing sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10% glycerol, 0.025% w/v bromphenol blue, 10% .beta.-mercaptoethanol), heated at 95.degree. C. for 10 min and separated on a 1 mm thick 5% SDS-polyacrylamide gel. [K. Weber and M. Osborn, "Proteins and Sodium Dodecyl Sulfate: Molecular Weight Determination on Polyacrylamide Gels and Related Procedures,"

in The Proteins, 3d Edition (H. Neurath & R. L. Hill, eds), pp. 179-223, (Academic Press, NY, 1975).] Part of the gel was cut off and the proteins were stained with Coomassie Blue. The proteins in the remainder of the gel were transferred to nitrocellulose using the Milliblot-SDE electro-blotting system (Millipore) according to manufacturer's directions. The nitrocellulose was temporarily stained with 10% Ponceau S [S. B. Carroll and A. Laughon, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of .beta.-galactosidase Fusion Proteins," in DNA Cloning: A Practical Approach, Vol. III, (D. Glover, ed.), pp. 89-111, IRL Press, Oxford, (1987)] to visualize the lanes, then destained by running a gentle stream of distilled water over the blot for several minutes. The nitrocellulose was immersed in PBS containing 3% BSA overnight at 4.degree. C. to block any remaining protein binding sites.

Detailed Description Text (181):
The blot was cut into strips and each strip was incubated with the appropriate primary antibody. The avian anti-C. botulinum antibodies [described in (c)] and preimmune chicken antibody (as control) were diluted 1:125 in PBS containing 1 mg/ml BSA for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS, BBS-Tween and PBS, successively (10 min/wash). Goat anti-chicken IgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:500 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS and BBS-Tween, followed by one change of PBS and 0.1M Tris-HCl, pH 9.5. Blots were developed in freshly prepared alkaline phosphatase substrate buffer (100 .mu.g/ml nitroblue tetrazolium (Sigma), 50 .mu.g/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 5 mM MgCl.sub.2 in 50 mM Na.sub.2 CO.sub.3, pH 9.5).

Detailed Description Text (182):
The Western blots are shown in FIG. 1. The anti-C. botulinum IgY reacted to the toxoid to give a broad immunoreactive band at about 145-150 kD on the reducing gel. This toxoid is refractive to disulfide cleavage by reducing agents due to formalin crosslinking. The immune IgY reacted with the active toxin complex, a 97 kD C. botulinum type A heavy chain and a 53 kD light chain. The preimmune IgY was unreactive to the C. botulinum complex or toxoid in the Western blot.

Detailed Description Text (184):
The IgY antibody titer to C. botulinum type A toxoid of eggs harvested between day 409 and 423 was evaluated by ELISA, prepared as follows. Ninety-six-well Falcon Pro-bind plates were coated overnight at 4.degree. C. with 100 .mu.l/well toxoid [B. R. Singh & B. R. Das Gupta, Toxicon 27:403 (1989)] at 2.5 .mu.g/ml in PBS, pH 7.5 containing 0.005% thimerosal. The following day the wells were blocked with PBS containing 1% BSA for 1 hour at 37.degree. C. The IgY from immune or preimmune eggs was diluted in PBS containing 1% BSA and 0.05% Tween 20 mad the plates were incubated for 1 hour at 37.degree. C. The plates were washed three times with PBS containing 0.05% Tween 20 and three times with PBS alone. Alkaline phosphatase-conjugated goat-anti-chicken IgG (Fisher Biotech) was diluted 1:750 in PBS containing 1% BSA and 0.05% Tween 20, added to the plates, and incubated 1 hour at 37.degree. C. The plates were washed as before, and p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.05M Na.sub.2 CO.sub.3, pH 9.5, 10 mM MgCl.sub.2 was added.

Detailed Description Text (185):
The results are shown in FIG. 2. Chickens immunized with the toxoid generated high titers of antibody to the immunogen. Importantly, eggs from both immunized hens had significant anti-immunogen antibody titers as compared to preimmune control eggs. The anti-C. botulinum IgY possessed significant activity, to a dilution of 1:93,750 or greater.

Detailed Description Text (212):
In Vivo Neutralization Of Type C. botulinum Type A Neurotoxin By Avian Antitoxin Antibody

Detailed Description Text (213):
This example demonstrated the ability of PEG-purified antitoxin, collected as described in Example 3, to neutralize the lethal effect of C. botulinum neurotoxin type A in mice. To determine the oral lethal dose (LD.sub.100) of toxin A, groups of BALB/c mice were given different doses of toxin per unit body weight (average body weight of 24 grams). For oral administration, toxin A complex, which contains the neurotoxin associated with other non-toxin proteins was used. This complex is markedly more toxic than purified neurotoxin when given by the oral route. [I. Ohishi et al., Infect. Immun., 16:106 (1977).] C. botulinum toxin type A complex, obtained from Eric Johnson

(University Of Wisconsin, Madison) was 250 .mu.g/ml in 50 mM sodium citrate, pH 5.5, specific toxicity 3.times.10.sup.7 mouse LD.sub.50 /mg with parenteral administration. Approximately 40-50 ng/gm body weight was usually fatal within 48 hours in mice maintained on conventional food and water. When mice were given a diet ad libitum of only Enfamil.RTM. the concentration needed to produce lethality was approximately 2.5 times higher (125 ng/gm body weight). Botulinal toxin concentrations of approximately 200 ng/gm body weight were fatal in mice fed Enfamil.RTM. containing preimmune IgY (resuspended in Enfamil.RTM. at the original yolk volume).

Detailed Description Text (214):
The oral LD.sub.100 of C. botulinum toxin was also determined in mice that received known amounts of a mixture of preimmune IgY-Ensure.RTM. delivered orally through feeding needles. Using a 22 gauge feeding needle, mice were given 250 .mu.l each of a preimmune IgY-Ensure.RTM. mixture (preimmune IgY dissolved in 1/4 original yolk volume) 1 hour before and 1/2 hour and 5 hours after administering botulinal toxin. Toxin concentrations given orally ranged from approximately 12 to 312 ng/gm body weight (0.3 to 7.5 .mu.g per mouse). Botulinal toxin complex concentration of approximately 40 ng/gm body weight (1 .mu.g per mouse) was lethal in all mice in less than 36 hours.

Detailed Description Text (239):
In order to determine whether hens previously immunized with C. difficile toxoids could tolerate subsequent booster immunizations using native toxins, a single hen from group CTAB was immunized for a third time, this time using a mixture of the native toxin A and native toxin B described in section (a) above (these toxins were not formaldehyde-treated, and were used in their active form). This was done in order to increase the amount (titer) and affinity of specific antitoxin antibody produced by the hen over that achieved by immunizing with toxoids only. On day 62, 0.1 ml of a toxin mixture was prepared which contained 200 .mu.g of native toxin A and 200 .mu.g of native toxin B. This toxin mixture was then emulsified in 0.1 ml of Titer Max adjuvant. A single CTAB hen was then immunized with the resulting immunogen emulsion, via two I.M. injections of 100 .mu.l each, into the breast area. This hen was marked with a wing band, and observed for adverse effects for a period of approximately 1 week, after which time the hen appeared to be in good health.

Detailed Description Text (245):
The toxin neutralization activity of the IgY solutions prepared in section (c) above was determined using an assay system that was modified from published methods. [Ehrich et al., Infect. Immun. 28:1041-1043 (1992); and McGee et al. Microb. Path. 12:333-341 (1992).] As additional controls, affinity-purified goat anti-C. difficile toxin A (Tech Lab) and affinity-purified goat anti-C. difficile toxin B (Tech Lab) were also assayed for toxin neutralization activity.

Detailed Description Text (247):
Unlike previous reports which quantitate results visually by counting cell rounding by microscopy, this Example utilized spectrophotometric methods to quantitate the C. difficile toxin bioassay. In order to determine the toxin A neutralizing activity of the CTA, CTAB, and pre-immune IgY preparations, as well as the affinity-purified goat antitoxin A control, dilutions of these antibodies were reacted against a 0.1 .mu.g/ml concentration of native toxin A (this is the approx. 50% cytotoxic dose of toxin A in this assay system). The results are shown in FIG. 3.

Detailed Description Text (248):
Complete neutralization of toxin A occurred with the CTA IgY (antitoxin A, above) at dilutions of 1:80 and lower, while significant neutralization occurred out to the 1:320 dilution. The CTAB IgY (antitoxin A+toxin B, above) demonstrated complete neutralization at the 1:320-1:160 and lower dilutions, and significant neutralization occurred out to the 1:1280 dilution. The commercially available affinity-purified goat antitoxin A did not completely neutralize toxin A at any of the dilutions tested, but demonstrated significant neutralization out to a dilution of 1:1,280. The preimmune IgY did not show any toxin A neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin A alone, or simultaneously with toxin A and toxin B, is an effective toxin A antitoxin.

Detailed Description Text (249):
The toxin B neutralizing activity of the CTAB and pre-immune IgY preparations, and also the affinity-purified goat antitoxin B control was determined by reacting dilutions of these antibodies against a concentration of native toxin B of 0.1 ng/ml (approximately the 50% cytotoxic dose of toxin B in the assay system). The results are shown in FIG.

4.

Detailed Description Text (250):
Complete neutralization of toxin B occurred with the CTAB IgY (antitoxin A+toxin B, above) at the 1:40 and lower dilutions, and significant neutralization occurred out to the 1:320 dilution. The affinity-purified goat antitoxin B demonstrated complete neutralization at dilutions of 1:640 and lower, and significant neutralization occurred out to a dilution of 1:2,560. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized simultaneously with toxin A and toxin B is an effective toxin B antitoxin.

Detailed Description Text (290):
To determine whether high levels of recombinant toxin A protein can be produced in E. coli, fragments of the toxin A gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin A protein in E. coli. Three prokaryotic expression systems were utilized. These systems were chosen because they drive expression of either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in E. coli, and allow affinity purification of the expressed protein on a ligand containing column. Fusion proteins expressed from pGEX vectors bind glutathione agarose beads, and are eluted with reduced glutathione. pMAL fusion proteins bind amylose resin, and are eluted with maltose. A poly-histidine tag is present at either the N-terminal (pET16b) or C-terminal (pET23a-c) end of pET fusion proteins. This sequence specifically binds Ni.sub.2.sup.+ chelate columns, and is eluted with imidazole salts. Extensive descriptions of these vectors are available (Williams et al. (1994) DNA Cloning: Expression Systems, in press), and will not be discussed in detail here. The Example involved (a) cloning of the toxin A gene, (b) expression of large fragments of toxin A in various prokaryotic expression systems, (c) identification of smaller toxin A gene fragments that express efficiently in E. coli, (d) purification of recombinant toxin A protein by affinity chromatography, and (e) demonstration of functional activity of a recombinant fragment of the toxin A gene.

Detailed Description Text (292):
A restriction map of the toxin A gene is shown in FIG. 6. The encoded protein contains a carboxy terminal ligand binding region, containing multiple repeats of a carbohydrate binding domain. [von Eichel-Streiber and Sauerborn, Gene 96:107-113 (1990).] The toxin A gene was cloned in three pieces, by using either the polymerase chain reaction (PCR) to amplify specific regions, (regions 1 and 2, FIG. 6) or by screening a constructed genomic library for a specific toxin A gene fragment (region 3, FIG. 6). The sequences of the utilized PCR primers are P1: 5' GGAAATT TAGCTGCAGCATCTGAC 3' (SEQ ID NO:1); P2: 5' TCTAGCAAATTCGCTTGT GTTGAA 3' (SEQ ID NO:2); P3: 5' CTCGCATATAGCATTAGACC 3' (SEQ ID NO:3); and P4: 5' CTATCTAGGCCTAAAGTAT 3' (SEQ ID NO:4). These regions were cloned into prokaryotic expression vectors that express either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in E. coli, and allow affinity purification of the expressed protein on a ligand containing column.

Detailed Description Text (294):
Fragments 1 and 2 were cloned by PCR, utilizing a proofreading thermostable DNA polymerase (native pfu polymerase; Stratagene). The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (e.g., Taq polymerase). PCR amplification was performed using the indicated PCR primers (FIG. 6) in 50 .mu.l reactions containing 10 mM Tris-HCl(8.3), 50 mM KCl, 1.5 mM MgCl.sub.2, 200 .mu.M each dNTP, 0.2 .mu.M each primer, and 50 ng C. difficile genomic DNA. Reactions were overlaid with 100 .mu.l mineral oil, heated to 94.degree. C. for 4 min, 0.5 .mu.l native pfu polymerase (Stratagene) added, and the reaction cycled 30.times. at 94.degree. C. for 1 min, 50.degree. C. for 1 min, 72.degree. C. for 4 min, followed by 10 min at 72.degree. C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 .mu.l TE buffer [10 mM Tris-HCL, 1 mM EDTA pH 8.0]. Aliquots of 10 .mu.l each were restriction digested with either EcoRI/HincII (fragment 1) or EcoRI/PstI (fragment 2), and the appropriate restriction fragments were gel purified using the Prep-A-Gene kit (BioRad), and ligated to either EcoRI/SmaI-restricted pGEX2T (Pharmacia) vector (fragment 1), or the EcoRI/PstI pMAlc (New England Biolabs) vector (fragment 2). Both clones are predicted to produce in-frame fusions with either the glutathione-S-transferase protein (pGEX vector) or the maltose binding protein (pMAL vector). Recombinant clones were isolated, and confirmed by restriction digestion, using standard recombinant molecular biology techniques. [Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), and designated pGA30-660 and pMA660-1100, respectively (see FIG. 6 for description of the clone designations).]

Detailed Description Text (295):
Fragment 3 was cloned from a genomic library of size selected PstI digested C. difficile genomic DNA, using standard molecular biology techniques (Sambrook et al.). Given that the fragment 3 internal PstI site is protected from cleavage in C. difficile genomic DNA [Price et al., Curr. Microbiol., 16:55-60 (1987)], a 4.7 kb fragment from PstI restricted C. difficile genomic DNA was gel purified, and ligated to PstI restricted, phosphatase treated pUC9 DNA. The resulting genomic library was screened with a oligonucleotide primer specific to fragment 3, and multiple independent clones were isolated. The presence of fragment 3 in several of these clones was confirmed by restriction digestion, and a clone of the indicated orientation (FIG. 6) was restricted with BamHI/HindIII, the released fragment purified by gel electrophoresis, and ligated into similarly restricted pET23c expression vector DNA (Novagert). Recombinant clones were isolated, and confirmed by restriction digestion. This construct is predicted to create both a predicted in frame fusion with the pET protein leader sequence, as well as a predicted C-terminal poly-histidine affinity tag, and is designated pPA1100-2680 (see FIG. 6 for the clone designation).

Detailed Description Text (297):
Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction, SDS-PAGE, and Western blot analysis are described in detail in Williams et al (1994), supra. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5+100 .mu.g/ml ampicillin were added to cultures of bacteria (BL21 for pMAl and pGEX plasmids, and BL21 (DE3)LysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37.degree. C., and induced when the cell density reached 0.5 OD.sub.600. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 .mu.l of 2.times. SDS-PAGE sample buffer [Williams et al. (1994), supra]. The samples were heated to 95.degree. C. for 5 min, the cooled and 5 or 10 .mu.l aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in FIG. 7. In this figure, lanes 1-3 contain cell lysates prepared from E. coli strains containing pPA1100-2860 in B121(DE3)lysE cells; lanes 4-6 contain cell lysates prepared from E. coli strains containing pPA1100-2860 in B121(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from E. coli strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from E. coli strains containing pMA660-1100. The lanes were probed with an affinity purified goat antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

Detailed Description Text (298):
Each construct directs expression of high molecular weight (HMW) protein that is reactive with the toxin A antibody. The size of the largest immunoreactive bands from each sample is consistent with predictions of the estimated MW of the intact fusion proteins. This demonstrates that the three fusions are in-frame, and that none of the clones contain cloning artifacts that disrupt the integrity of the encoded fusion protein. However, the Western blot demonstrates that fusion protein from the two larger constructs (pGA30-660 and pPA1100-2680) are highly degraded. Also, expression levels of toxin A proteins from these two constructs are low, since induced protein bands are not visible by Coomassie staining (not shown). Several other expression constructs that fuse large sub-regions of the toxin A gene to either pMALc or pET23a-c expression vectors, were constructed and tested for protein induction. These constructs were made by mixing gel purified restriction fragments, derived from the expression constructs shown in FIG. 6, with appropriately cleaved expression vectors, ligating, and selecting recombinant clones in which the toxin A restriction fragments had ligated together and into the expression vector as predicted for in-frame fusions. The expressed toxin A interval within these constructs are shown in FIG. 8, as well as the internal restriction sites utilized to make these constructs.

Detailed Description Text (300):
In all cases, Western blot analysis of each of these constructs with goat antitoxin A antibody (Tech Lab) detected HMW fusion protein of the predicted size (not shown). This confirms that the reading frame of each of these clones is not prematurely terminated, and is fused in the correct frame with the fusion partner. However, the Western blot analysis revealed that in all cases, the induced protein is highly degraded, and, as assessed by the absence of identifiable induced protein bands by Coomassie Blue staining, are expressed only at low levels. These results suggest that expression of high levels of intact toxin A recombinant protein is not possible when large regions of the toxin A gene are expressed in E. coli using these expression vectors.

Detailed Description Text (301):
c) High Level Expression Of Small Toxin A Protein Fusions In E. coli

Detailed Description Text (302):
Experience indicates that expression difficulties are often encountered when large (greater than 100 kd) fragments are expressed in E. coli. A number of expression constructs containing smaller fragments of the toxin A gene were constructed, to determine if small regions of the gene can be expressed to high levels without extensive protein degradation. A summary of these expression constructs are shown in FIG. 9. All were constructed by in-frame fusions of convenient toxin A restriction fragments to either the pMALc or pET23a-c vectors. Protein preparations from induced cultures of each of these constructs were analyzed by both Coomassie Blue staining and Western analysis as in (b) above. In all cases, higher levels of intact, full length fusion proteins were observed than with the larger recombinants from section (b).

Detailed Description Text (304):
Large scale (500 ml) cultures of each recombinant from (c) were grown, induced, and soluble and insoluble protein fractions were isolated. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams et al. (1994), supra]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts as described by the distributor (Novagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in column buffer (10 mM NaPO4, 0.5M NaCl, 10 mM .beta.-mercaptoethanol, pH 7.2) over an amylose resin column (New England Biolabs), and eluted with column buffer containing 10 mM maltose as described [Williams et al. (1994), supra]. When the expressed protein was found to be predominantly insoluble, insoluble protein extracts were prepared by the method described in Example 17, infra. The results are summarized in Table 16. FIG. 10 shows the sample purifications of recombinant toxin A protein. In this figure, lanes 1 and 2 contain MBP fusion protein purified by affinity purification of soluble protein.

Detailed Description Text (305):
Lanes 3 and 4 contain MBP fusion protein purified by solubilization of insoluble inclusion bodies. The purified fusion protein samples are pMA1870-2680 (lane 1), pMA660-1100 (lane 2), pMA300-600 (lane 3) and pMA1450-1870 (lane 4).

Detailed Description Text (306):
Poor yields of affinity purified protein were obtained when poly-histidine tagged pET vectors were used to drive expression (pPA1100-1450, pP1100-1870). However, significant protein yields were obtained from pMAL expression constructs spanning the entire toxin A gene, and yields of full-length soluble fusion protein ranged from an estimated 200-400 .mu.g/500 ml culture (pMA30-300) to greater than 20 mg/500 ml culture (pMA1610-1870). Only one interval was expressed to high levels as strictly insoluble protein (pMA300-660). Thus, although high level expression was not observed when using large expression constructs from the toxin A gene, usable levels of recombinant protein spanning the entire toxin A gene were obtainable by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin A gene. This is the first demonstration of the feasibility of expressing recombinant toxin A protein to high levels in E. coli.

Detailed Description Text (308):
The carboxy terminal end consisting of the repeating units contains the hemagglutination activity or binding domain of C. difficile toxin A. To determine whether the expressed toxin A recombinants retain functional activity, hemagglutination assays were performed. Two toxin A recombinant proteins, one containing the binding domain as either soluble affinity purified protein (pMA1870-2680) or SDS solubilized inclusion body protein (pPA1870-2680) and soluble protein from one region outside that domain (pMA1100-1610) were tested using a described procedure. [H. C. Krivan el. al.,

Infect. Immun., 53:573 (1986).] Citrated rabbit red blood cells (RRBC)(Cocalico) were washed several times with Tris-buffer (0.1M Tris and 50 mM NaCl) by centrifugation at 450.times.g for 10 minutes at 4.degree. C. A 1% RRBC suspension was made from the packed cells and resuspended in Tris-buffer. Dilutions of the recombinant proteins and native toxin A (Tech Labs) were made in the Tris-buffer and added in duplicate to a round-bottomed 96-well microtiter plate in a final volume of 100 .mu.l. To each well, 50 .mu.l of the 1% RRBC suspension was added, mixed by gentle tapping, and incubated at 4.degree. C. for 3-4 hours. Significant hemagglutination occurred only in the recombinant proteins containing the binding domain (pMA 1870-2680) and native toxin A. The recombinant protein outside the binding domain (pMA 1100-1610) displayed no hemagglutination activity. Using equivalent protein concentrations, the hemagglutination titer for toxin A was 1:256, while titers for the soluble and insoluble recombinant proteins of the binding domain were 1:256 and about 1:5000. Clearly, the recombinant proteins tested retained functional activity and were able to bind RRBC's.

Detailed Description Text (311):
The expression of recombinant toxin A protein as multiple fragments in E. coli has demonstrated the feasibility of generating toxin A antigen through use of recombinant methodologies (Example 11). The isolation of these recombinant proteins allows the immunoreactivity of each individual subregion of the toxin A protein to be determined (i.e., in a antibody pool directed against the native toxin A protein). This identifies the regions (if any) for which little or no antibody response is elicited when the whole protein is used as a immunogen. Antibodies directed against specific fragments of the toxin A protein can be purified by affinity chromatography against recombinant toxin A protein, and tested for neutralization ability. This identifies any toxin A subregions that are essential for producing neutralizing antibodies. Comparison with the levels of immune response directed against these intervals when native toxin is used as an immunogen predicts whether potentially higher titers of neutralizing antibodies can be produced by using recombinant protein directed against a individual region, rather than the entire protein. Finally, since it is unknown whether antibodies reactive to the recombinant toxin A proteins produced in Example 11 neutralize toxin A as effectively as antibodies raised against native toxin A (Examples 9 and 10), the protective ability of a pool of antibodies affinity purified against recombinant toxin A fragments was assessed for its ability to neutralize toxin A.

Detailed Description Text (312):
This Example involved (a) epitope mapping of the toxin A protein to determine the titre of specific antibodies directed against individual subregions of the toxin A protein when native toxin A protein is used as an immunogen, (b) affinity purification of IgY reactive against recombinant proteins spanning the toxin A gene, (c) toxin A neutralization assays with affinity purified IgY reactive to recombinant toxin A protein to identify subregions of the toxin A protein that induce the production of neutralizing antibodies, and determination of whether complete neutralization of toxin A can be elicited with a mixture of antibodies reactive to recombinant toxin A protein.

Detailed Description Text (314):
The affinity purification of recombinant toxin A protein specific to defined intervals of the toxin A protein allows epitope mapping of antibody pools directed against native toxin A. This has not previously been possible, since previous expression of toxin A recombinants has been assessed only by Western blot analysis, without knowledge of the expression levels of the protein [e.g., von Eichel-Streiber et al, J. Gen. Microbiol., 135:55-64 (1989)]. Thus, high or low reactivity of recombinant toxin A protein on Western blots may reflect protein expression level differences, not immunoreactivity differences. Given that the purified recombinant protein generated in Example 11 have been quantitated, the issue of relative immunoreactivity of individual regions of the toxin A protein was precisely addressed.

Detailed Description Text (318):
b) Affinity Purification Of IgY Reactive Against Recombinant Toxin A Protein

Detailed Description Text (319):
Affinity columns, containing recombinant toxin A protein from the 6 defined intervals in (a) above, were made and used to (i) affinity purify antibodies reactive to each individual interval from the CTA IgY preparation [Example 8(c)], and (ii) deplete interval specific antibodies from the CTA IgY preparation. Affinity columns were made by coupling 1 ml of PBS-washed Actigel resin (Sterogene) with region specific protein and 1/10 final volume of Ald-coupling solution (1M sodium cyanoborohydride). The total

region specific protein added to each reaction mixture was 2.7 mg (interval 1), 3 mg (intervals 2 and 3), 0.1 mg (interval 4), 0.2 mg (interval 5) and 4 mg (interval 6). Protein for intervals 1, 3, and 6 was affinity purified pMAl fusion protein in column buffer (see Example 11). Interval 4 was affinity purified poly-histidine containing pET fusion in PBS; intervals 2 and 5 were from inclusion body preparations of insoluble pMAL fusion protein, dialyzed extensively in PBS. Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 50% coupling efficiencies were estimated. The resins were poured into 5 ml BioRad columns, washed extensively with PBS, and stored at 4.degree. C.

Detailed Description Text (320):
Aliquots of the CTA IgY polyclonal antibody preparation were depleted for each individual region as described below. A 20 ml sample of the CTA IgY preparation [Example 8(c)] was dialyzed extensively against 3 changes of PBS (1 liter for each dialysis), quantitated by absorbance at OD.sub.280, and stored at 4.degree. C. Six 1 ml aliquots of the dialyzed IgY preparation were removed, and depleted individually for each of the six intervals. Each 1 ml aliquot was passed over the appropriate affinity column, and the eluate twice reapplied to the column. The eluate was collected, and pooled with a 1 ml PBS wash. Bound antibody was eluted from the column by washing with 5 column volumes of 4M Guanidine-HCl (in 10 mM Tris-HCl, pH 8.0). The column was reequilibrated in PBS, and the depleted antibody stock reapplied as described above. The eluate was collected, pooled with a 1 ml PBS wash, quantitated by absorbance at OD.sub.280, and stored at 4.degree. C. In this manner, 6 aliquots of the CTA IgY preparation were individually depleted for each of the 6 toxin A intervals, by two rounds of affinity depletion. The specificity of each depleted stock was tested by Western blot analysis. Multiple 7.5% SDS-PAGE gels were loaded with protein samples corresponding to all 6 toxin A subregions. After electrophoresis, the gels were blotted, and protein transfer confirmed by Ponceau S staining [protocols described in Williams et al. (1994), supra]. After blocking the blots 1 hr at 20.degree. C. in PBS+0.1% Tween 20 (PBST) containing 5% milk (as a blocking buffer), 4 ml of either a 1/500 dilution of the dialyzed CTA IgY preparation in blocking buffer, or an equivalent amount of the six depleted antibody stocks (using OD.sub.280 to standardize antibody concentration) were added and the blots incubated a further 1 hr at room temperature. The blots were washed and developed with alkaline phosphatase (using a rabbit anti-chicken alkaline phosphate conjugate as a secondary antibody) as previously described [Williams et al. (1994), supra]. In all cases, only the target interval was depleted for antibody reactivity, and at least 90% of the reactivity to the target intervals was specifically depleted.

Detailed Description Text (321):
Region specific antibody pools were isolated by affinity chromatography as described below. Ten mls of the dialyzed CTA IgY preparation were applied sequentially to each affinity column, such that a single 10 ml aliquot was used to isolate region specific antibodies specific to each of the six subregions. The columns were sequentially washed with 10 volumes of PBS, 6 volumes of BBS-Tween, 10 volumes of TBS, and eluted with 4 ml Actisep elution media (Sterogene). The eluate was dialyzed extensively against several changes of PBS, and the affinity purified antibody collected and stored at 4.degree. C. The volumes of the eluate increased to greater than 10 mls during dialysis in each case, due to the high viscosity of the Actisep elution media. Aliquots of each sample were 20.times. concentrated using Centricon 30 microconcentrators (Amicon) and stored at 4.degree. C. The specificity of each region specific antibody pool was tested, relative to the dialyzed CTA IgY preparation, by Western blot analysis, exactly as described above, except that 4 ml samples of blocking buffer containing 100 .mu.l region specific antibody (unconcentrated) were used instead of the depleted CTA IgY preparations. Each affinity purified antibody preparation was specific to the defined interval, except that samples purified against intervals 1-5 also reacted with interval 6. This may be due to non-specific binding to the interval 6 protein, since this protein contains the repetitive ligand binding domain which has been shown to bind antibodies nonspecifically. [Lyerly et al., Curr. Microbiol., 19:303-306 (1989).]

Detailed Description Text (322):
The reactivity of each affinity purified antibody preparation to the corresponding proteins was approximately the same as the reactivity of the 1/500 diluted dialyzed CTA IgY preparation standard. Given that the specific antibody stocks were diluted 1/40, this would indicate that the unconcentrated affinity purified antibody stocks contain 1/10-1/20 the concentration of specific antibodies relative to the starting CTA IgY preparation.

Detailed Description Text (324):
The CHO toxin neutralization assay [Example 8(d)] was used to assess the ability of the depleted or enriched samples generated in (b) above to neutralize the cytotoxicity of toxin A. The general ability of affinity purified antibodies to neutralize toxin A was assessed by mixing together aliquots of all 6 concentrated stocks of the 6 affinity purified samples generated in (b) above, and testing the ability of this mixture to neutralize a toxin A concentration of 0.1 .mu.g/ml. The results, shown in FIG. 11, demonstrate almost complete neutralization of toxin A using the affinity purified (AP) mix. Some epitopes within the recombinant proteins utilized for affinity purification were probably lost when the proteins were denatured before affinity purification [by Guanidine-HCl treatment in (b) above]. Thus, the neutralization ability of antibodies directed against recombinant protein is probably underestimated using these affinity purified antibody pools. This experiment demonstrates that antibodies reactive to recombinant toxin A can neutralize cytotoxicity, suggesting that neutralizing antibodies may be generated by using recombinant toxin A protein as immunogen.

Detailed Description Text (326):
In the toxin neutralization experiment shown in FIG. 11, interval 6 specific antibodies (interval 6 contains the ligand binding domain) were depleted from the dialyzed PEG preparation, and the effect on toxin neutralization assayed. Interval 6 antibodies were depleted either by utilizing the interval 6 depleted CTA IgY preparation from (b) above ("-6 aff. depleted" in FIG. 11), or by addition of interval 6 protein to the CTA IgY preparation (estimated to be a 10 fold molar excess over anti-interval 6 immunoglobulin present in this preparation) to competitively compete for interval 6 protein ("-6 prot depleted" in FIG. 11). In both instances, removal of interval 6 specific antibodies reduces the neutralization efficiency relative to the starting CTA IgY preparation. This demonstrates that antibodies directed against interval 6 contribute to toxin neutralization. Since interval 6 corresponds to the ligand binding domain of the protein, these results demonstrate that antibodies directed against this region in the PEG preparation contribute to the neutralization of toxin A in this assay. However, it is significant that after removal of these antibodies, the PEG preparation retains significant ability to neutralize toxin A (FIG. 11). This neutralization is probably due to the action of antibodies specific to other regions of the toxin A protein, since at least 90% of the ligand binding region reactive antibodies were removed in the depleted sample prepared in (b) above. This conclusion was supported by comparison of the toxin neutralization of the affinity purified (AP) mix compared to affinity purified interval 6 antibody alone. Although some neutralization ability was observed with AP interval 6 antibodies alone, the neutralization was significantly less than that observed with the mixture of all 6 AP antibody stocks (not shown).

Detailed Description Text (327):
Given that the mix of all six affinity purified samples almost completely neutralized the cytotoxicity of toxin A (FIG. 11), the relative importance of antibodies directed against toxin A intervals 1-5 within the mixture was determined. This was assessed in two ways. First, samples containing affinity purified antibodies representing 5 of the 6 intervals were prepared, such that each individual region was depleted from one sample. FIG. 12 demonstrates a sample neutralization curve, comparing the neutralization ability of affinity purified antibody mixes without interval 4 (-4) or 5 (-5) specific antibodies, relative to the mix of all 6 affinity purified antibody stocks (positive control). While the removal of interval 5 specific antibodies had no effect on toxin neutralization (or intervals 1-3, not shown), the loss of interval 4 specific antibodies significantly reduced toxin neutralization (FIG. 12).

Detailed Description Text (328):
Similar results were seen in a second experiment, in which affinity purified antibodies, directed against a single region, were added to interval 6 specific antibodies, and the effects on toxin neutralization assessed. Only interval 4 specific antibodies significantly enhanced neutralization when added to interval 6 specific antibodies (FIG. 13). These results demonstrate that antibodies directed against interval 4 (corresponding to clone pPA1100-1450 in FIG. 9) are important for neutralization of cytotoxicity in this assay. Epitope mapping has shown that only low levels of antibodies reactive to this region are generated when native toxin A is used as an immunogen [Example 12(a)]. It is hypothesized that immunization with recombinant protein specific to this interval will elicit higher titers of neutralizing antibodies. In summary, this analysis has identified two critical regions of the toxin A protein against which neutralizing antibodies are produced, as assayed by the CHO neutralization assay.

Detailed Description Text (331):

In Example 12, we demonstrated neutralization of toxin A mediated cytotoxicity by affinity purified antibodies reactive to recombinant toxin A protein. To determine whether antibodies raised against a recombinant polypeptide fragment of C. difficile toxin A may be effective in treating clostridial diseases, antibodies to recombinant toxin A protein representing the binding domain were generated. Two toxin A binding domain recombinant polypeptides, expressing the binding domain in either the pMALc (pMA1870-2680) or pET 23(pPA1870-2680) vector, were used as immunogens. The pMAL protein was affinity purified as a soluble product [Example 12(d)] and the pET protein was isolated as insoluble inclusion bodies [Example 12(d)] and solubilized to an immunologically active protein using a proprietary method described in a pending patent application (U.S. patent application Ser. No. 08/129,027). This Example involves (a) immunization, (b) antitoxin collection, (c) determination of antitoxin antibody titer, (d) anti-recombinant toxin A neutralization of toxin A hemagglutination activity in vitro, and (e) assay of in vitro toxin A neutralizing activity.

Detailed Description Text (337):
To determine if the recombinant toxin A protein was sufficiently immunogenic to raise antibodies in hens, the antibody titer of a recombinant toxin A polypeptide was determined by ELISA. Eggs from both hens were collected on day 32, the yolks pooled and the antibody was isolated using PEG as described. The immune recombinant IgY antibody titer was determined for the soluble recombinant protein containing the maltose binding protein fusion generated in p-Mal (pMA1870-2680). Ninety-six well Falcon Pro-bind plates were coated overnight at 4.degree. C. with 100 .mu.l/well of toxin A recombinant at 2.5 .mu.g/.mu.l in PBS containing 0.05% thimerosal. Another plate was also coated with maltose binding protein (MBP) at the same concentration, to permit comparison of antibody reactivity to the fusion partner. The next day, the wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37.degree. C. IgY isolated from immune or preimmune eggs was diluted in antibody diluent (PBS containing 1% BSA and 0.05% Tween-20), and added to the blocked wells and incubated for 1 hour at 37.degree. C. The plates were washed three times with PBS with 0.05% Tween-20, then three times with PBS. Alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) diluted 1:1000 in antibody diluent was added to the plate, and incubated for 1 hour at 37.degree. C. The plates were washed as before and substrate was added, [p-nitrophenyl phosphate (Sigma)] at 1 mg/ml in 0.05M Na.sub.2 CO.sub.3, pH 9.5 and 10 mM MgCl.sub.2. The plates were evaluated quantitatively on a Dynatech MR 300 Micro EPA plate reader at 410 nm about 10 minutes after the addition of substrate.

Detailed Description Text (338):
Based on these ELISA results, high antibody titers were raised in chickens immunized with the toxin A recombinant polypeptide. The recombinant appeared to be highly immunogenic, as it was able to generate high antibody titers relatively quickly with few immunizations. Immune IgY titer directed specifically to the toxin A portion of the recombinant was higher than the immune IgY titer to its fusion partner, the maltose binding protein, and significantly higher than the preimmune IgY. ELISA titers (reciprocal of the highest dilution of IgY generating a signal) in the preimmune IgY to the MBP or the recombinant was <1:30 while the immune IgY titers to MBP and the toxin A recombinant were 1:18750 and >1:93750 respectively. Importantly, the anti-recombinant antibody titers generated in the hens against the recombinant polypeptide is much higher, compared to antibodies to that region raised using native toxin A. The recombinant antibody titer to region 1870-2680 in the CTA antibody preparation is at least five-fold lower compared to the recombinant generated antibodies (1:18750 versus >1:93750). Thus, it appears a better immune response can be generated against a specific recombinant using that recombinant as the immunogen compared to the native toxin A.

Detailed Description Text (376):
To determine the concentration of Interval 6-specific antibodies (expressed as percent of total antibody) within the CTA and pMA1870-2680 (Interval 6) PEG preparations, defined quantities of these antibody preparations were affinity purified on a pPA1870-2680(H) (shown schematically in FIG. 15B) affinity column and the specific antibodies were quantified. In FIG. 15B the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMAL.TM.-c vector (New England BioLabs); pG refers to the pGEX vector (Pharmacia); pB refers to the PinPoint.TM. Xa vector (Promega); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; the hatched ovals represent glutathione S-transferase; the hatched circles represent the biotin tag; and HHH represents the poly-histidine tag.

Detailed Description Text (377):

An affinity column containing recombinant toxin A repeat protein was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5-10 mg of pPA1870-2680 inclusion body protein [prepared as described in Example (17) and dialyzed into PBS] in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based upon protein band intensities, greater than 6 mg of recombinant protein was coupled to the resin. The resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated with PBS. The column was stored at 4.degree. C.

Detailed Description Text (378):
Aliquots of a pMA1870-2680 (Interval 6) or a CTA IgY polyclonal antibody preparation (PEG prep) were affinity purified on the above affinity column as follows. The column was attached to an UV monitor (ISCO) and washed with PBS. For pMA1870-2680 IgY purification, a 2X PEG prep (filter sterilized using a 0.45.mu. filter; approximately 500 mg total IgY) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal). The entire elution peak was collected in a 15 ml tube (Falcon). The column was re-equilibrated and the column eluate was re-chromatographed as described above. The antibody preparation was quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Total purified antibody was approximately 9 mg and 1 mg from the first and second chromatography passes, respectively. The low yield from the second pass indicated that most specific antibodies were removed by the first round of chromatography. The estimated percentage of Interval 6 specific antibodies in the pMA1870-2680 PEG prep is approximately 2%.

Detailed Description Text (394):
The animals were dosed daily at roughly 4 hr intervals with 1 ml antibody preparation diluted in Ensure.RTM. (Ross Labs). The amount of specific antibodies given per dose (determined by affinity purification) was estimated to be about 400 .mu.g of anti-Interval 6 IgY (for animals in the Interval 6 group) and 100 .mu.g and 70 .mu.g of anti-toxin A (Interval 6-specific) and anti-toxin B (Interval 3-specific; see Example 19), respectively, for the CTAB preparation. The animals were treated for 9 days and then observed for an additional 4 days for the presence of diarrhea and death. The results indicating the number of survivors and the number of dead 4 days post-infection are shown in Table 20.

Detailed Description Text (401):
The interval 6 immunogen utilized in Examples 15 and 16 was the pMA1870-2680 protein, in which the toxin A repeats are expressed as a soluble fusion protein with the MBP (described in Example 11). Interestingly, expression of this region (from the SpeI site to the end of the repeats, see FIG. 15B) in three other expression constructs, as either native (pPA1870-2680), poly-His tagged [pPA1870-2680 (H)] or biotin-tagged (pBA1870-2680) proteins resulted in completely insoluble protein upon induction of the bacterial host (see FIG. 15B). The host strain BL21 (Novagen) was used for expression of pBA1870-2680 and host strain BL21(DE3) (Novagen) was used for expression of pPA1870-2680 and pPA1870-2680(H). These insoluble proteins accumulated to high levels in inclusion bodies. Expression of recombinant plasmids in E. coli host cells grown in 2X YT medium was performed as described [Williams, et al. (1994), supra].

Detailed Description Text (402):
As summarized in FIG. 15B, expression of fragments of the toxin A repeats (as either N-terminal SpeI-EcoRI fragments, or C-terminal EcoRI-end fragments) also yielded high levels of insoluble protein using pGEX (pGA1870-2190), PinPoint.TM.-Xa (pBA1870-2190 and pBA2250-2680) and pET expression systems (pPA1870-2190). The pGEX and pET expression systems are described in Example 11. The PinPoint.TM.-Xa expression system drives the expression of fusion proteins in E. coli. Fusion proteins from PinPoint.TM.-Xa vectors contain a biotin tag at the amino-terminal end and can be affinity purified SoftLink.TM. Soft Release avidin resin (Promega) under mild denaturing conditions (5 mM biotin).

Detailed Description Text (403):
The solubility of expressed proteins from the pPG 1870-2190 and pPA 1870-2190 expression constructs was determined after induction of recombinant protein expression under conditions reported to enhance protein solubility [These conditions comprise growth of the host at reduced temperature (30.degree. C.) and the utilization of high

(1 mM IPTG) or low (0.1 mM IPTG) concentrations of inducer [Williams et al. (1994), supra]. All expressed recombinant toxin A protein was insoluble under these conditions. Thus, expression of these fragments of the toxin A repeats in pET and pGEX expression vectors results in the production of insoluble recombinant protein even when the host cells are grown at reduced temperature and using lower concentrations of the inducer. Although expression of these fragments in pMal vectors yielded affinity purifiable soluble fusion protein, the protein was either predominantly insoluble (pMA1870-2190) or unstable (pMA2250-2650). Attempts to solubilize expressed protein from the pMA1870-2190 expression construct using reduced temperature or lower inducer concentration (as described above) did not improve fusion protein solubility.

Detailed Description Text (404):
Collectively, these results demonstrate that expression of the toxin A repeat region in E. coli results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or poly-his tagged pET, pGEX or PinPoint.TM.-Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

Detailed Description Text (435):
Total protein extracts were isolated, resolved on SDS-PAGE gels, and toxin B protein identified by Western analysis utilizing a goat anti-toxin B affinity purified antibody (Tech Lab). Procedures for protein induction, SDS-PAGE, and Western blot analysis were performed as described in Williams et al. (1994), supra. In brief, 5 ml cultures of bacteria grown in 2XYT containing 100 .mu.g/ml ampicillin containing the appropriate recombinant clone were induced to express recombinant protein by addition of IPTG to 1 mM. The E. coli hosts used were: BL21(DE3) or BL21(DE3)LysS (Novagen) for pET plasmids.

Detailed Description Text (436):
Cultures were induced by the addition of IPTG to a final concentration of 1.0 mM when the cell density reached 0.5 OD.sub.600, and induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in microfuge), and resuspension of the pelleted bacteria in 150 .mu.l of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; .beta.-mercaptoethanol is added to 5% before use). The samples were heated to 95.degree. C. for 5 min, then cooled and 5 or 10 .mu.ls loaded on 7.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining the gels with Coomassie Blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. The MW of induced toxin B reactive protein allowed the integrity of the toxin B reading frame to be determined.

Detailed Description Text (437):
The pET23b recombinant (pPB10-1530) expressed high MW recombinant toxin B reactive protein, consistent with predicted values. This confirmed that frame terminating errors had not occurred during PCR amplification. A pET23b expression clone containing the 10-1750aa interval of the toxin B gene was constructed, by fusion of the EcoRV-SpeI fragment of the P7/P8 amplification product to the P5-EcoRV interval of the P5/P6 amplification product (see FIG. 18) in pPB10-1530. The integrity of this clone (pPB10-1750) was confirmed by restriction mapping, and Western blot detection of expressed recombinant toxin B protein. Levels of induced protein from both pPB10-1530 and pPB10-1750 were too low to facilitate purification of usable amounts of recombinant toxin B protein. The remaining 1750-2360 aa interval was directly cloned into pMAL or pET expression vectors from the P7/P8 amplification product as described below.

Detailed Description Text (440):
Fragments of the toxin B gene were expressed as either native or fusion proteins in E. coli. Native proteins were expressed in either the pET23a-c or pET16b expression vectors (Novagen). The pET23 vectors contain an extensive polylinker sequence in all three reading frames (a-c vectors) followed by a C-terminal poly-histidine repeat. The pET16b vector contains a N-terminal poly-histidine sequence immediately 5' to a small polylinker. The poly-histidine sequence binds to Ni-Chelate columns and allows affinity purification of tagged target proteins [Williams et al. (1994), supra]. These affinity tags are small (10 aa for pET16b, 6 aa for pET23) allowing the expression and affinity purification of native proteins with only limited amounts of foreign sequences.

Detailed Description Text (442):
All MBP fusion proteins were constructed and expressed in the pMAL.TM.-c or pMAL.TM.-p2 vectors (New England Biolabs) in which the protein of interest is expressed as a C-terminal fusion with MBP. All pET plasmids were expressed in either the BL21(DE3) or BL21(DE3)LysS expression hosts, while pMal plasmids were expressed in the BL21 host.

Detailed Description Text (443):
Large scale (500 mls to 1 liter) cultures of each recombinant were grown in 2X YT broth, induced, and soluble protein fractions were isolated as described [Williams, et al. (1994), supra]. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams et al., (1994) supra]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts or low pH (pH 4.0) as described by the distributor (Novagen or Qiagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in PBS buffer over an amylose resin (New England Biolabs) column, and eluted with PBS containing 10 mM maltose as described [Williams et al. (1994), supra].

Detailed Description Text (445):
In both large expression constructs described in (a) above, only low level (i.e., less than 1 mg/liter of intact or nondegraded recombinant protein) expression of recombinant protein was detected. A number of expression constructs containing smaller fragments of the toxin B gene were then constructed, to determine if small regions of the gene can be expressed to high levels (i.e., greater than 1 mg/liter intact protein) without extensive protein degradation. All were constructed by in frame fusions of convenient toxin B restriction fragments to either the pMAL-c, pET23a-c, pET16b or pETHisa-b expression vectors, or by engineering restriction sites at specific locations using PCR amplification [using the same conditions described in (a) above]. In all cases, clones were verified by restriction mapping, and, where indicated, DNA sequencing.

Detailed Description Text (446):
Protein preparations from induced cultures of each of these constructs were analyzed, by SDS-PAGE, to estimate protein stability (Coomassie Blue staining) and immunoreactivity against anti-toxin B specific antiserum (Western analysis). Higher levels of intact (i.e., nondegraded), full length fusion proteins were observed with the smaller constructs as compared with the larger recombinants, and a series of expression constructs spanning the entire toxin B gene were constructed (FIGS. 18, 19 and 20 and Table 23).

Detailed Description Text (447):
Constructs that expressed significant levels of recombinant toxin B protein (greater than 1 mg/liter intact recombinant protein) in E. coli were identified and a series of these clones that spans the toxin B gene are shown in FIG. 19 and summarized in Table 23. These clones were utilized to isolate pure toxin B recombinant protein from the entire toxin B gene. Significant protein yields were obtained from pMAL expression constructs spanning the entire toxin B gene, and yields of full length soluble fusion protein ranged from an estimated 1 mg/liter culture (pMB1100-1530) to greater than 20 mg/liter culture (pMB1750-2360).

Detailed Description Text (448):
Representative purifications of MBP and poly-histidine-tagged toxin B recombinants are shown in FIGS. 21 and 22. FIG. 21 shows a Coomassie Blue stained 7.5% SDS-PAGE gel on which various protein samples extracted from bacteria harboring pMB1850-2360 were electrophoresed. Samples were loaded as follows: Lane 1: protein extracted from uninduced culture; Lane 2: induced culture protein; Lane 3: total protein from induced culture after sonication; Lane 4: soluble protein; and Lane 5: eluted affinity purified protein. FIG. 22 depicts the purification of recombinant proteins expressed in bacteria harboring either pPB1850-2360 (Lanes 1-3) or pPB1750-2360 (Lanes 4-6). Samples were loaded as follows: uninduced total protein (Lanes 1 and 4); induced total protein (Lanes 2 and 5); and eluted affinity purified protein (Lanes 3 and 6). The broad range molecular weight protein markers (BioRad) are shown in Lane 7.

Detailed Description Text (450):
These results represent the first demonstration of the feasibility of expressing recombinant toxin B protein to high levels in E. coli. As well, expression of small regions of the putative ligand binding domain (repeat region) of toxin B as native protein yielded insoluble protein, while large constructs, or fusions to MBP were soluble (FIG. 19), demonstrating that specific methodologies are necessary to produce soluble fusion protein from this interval.

Detailed Description Text (454):
pET and pMal clones containing the toxin B repeat insert (aa interval 1750-2360 of toxin B) were verified by restriction mapping, using enzymes that cleaved specific sites within the toxin B region. In both cases fusion of the toxin B SpeI site with either the compatible XbaI site (pMal) or compatible NheI site (pET) is predicted to create an in frame fusion. This was confirmed in the case of the pMB1750-2360 clone by DNA sequencing of the clone junction and 5' end of the toxin B insert using a MBP specific primer (New England Biolabs). In the case of the pET construct, the fusion of the blunt ended toxin B 3' end to the filled HindIII site should create an in-frame fusion with the C-terminal poly-histidine sequence in this vector. The pPB1750-2360 clone selected had lost, as predicted, the HindIII site at this clone junction; this eliminated the possibility that an additional adenosine residue was added to the 3' end of the PCR product by a terminal transferase activity of the Pfu polymerase, since fusion of this adenosine residue to the filled HindIII site would regenerate the restriction site (and was observed in several clones).

Detailed Description Text (455):
One liter cultures of each expression construct were grown, and fusion protein purified by affinity chromatography on either an amylose resin column (pMAL constructs; resin supplied by New England Biolabs) or Ni-chelate column (pET constructs; resin supplied by Qiagen or Novagen) as described [Williams et al. (1994), supra]. The integrity and purity of the fusion proteins were determined by Coomassie staining of SDS-PAGE protein gels as well as Western blot analysis with either an affinity purified goat polyclonal antiserum (Tech Lab), or a chicken polyclonal PEG prep, raised against the toxin B protein (CTB) as described above in Example 8. In both cases, affinity purification resulted in yields in excess of 20 mg protein per liter culture, of which greater than 90% was estimated to be full-length recombinant protein. It should be noted that the poly-histidine affinity tagged protein was released from the Qiagen Ni-NTA resin at low imidazole concentration (60 mM), necessitating the use of a 40 mM imidazole rather than a 60 mM imidazole wash step during purification.

Detailed Description Text (456):
A periplasmically secreted version of pMB1750-2360 was constructed by replacement of the promoter and MBP coding region of this construct with that from a related vector (pMAL.TM.-p2; New England Biolabs) in which a signal sequence is present at the N-terminus of the MBP, such that fusion protein is exported. This was accomplished by substituting a BglII-EcoRV promoter fragment from pMAL-p2 into pMB1750-2360. The yields of secreted, affinity purified protein (recovered from osmotic shock extracts as described by Riggs in Current Protocols in Molecular Biology, Vol. 2, Ausubel, et al., Eds. (1989), Current Protocols, pp. 16.6.1-16.6.14] from this vector (pMBp1750-2360) were 6.5 mg/liter culture, of which 50% was estimated to be full-length fusion protein.

Detailed Description Text (459):
No fusion protein was secreted in the pMB1970-2360 version, perhaps due to a conformational constraint that prevents export of the fusion protein. However, the intracellularly expressed vector produced greater than 40 mg/liter of greater than 90% full-length fusion protein.

Detailed Description Text (461):
The amplified fragment was cloned into the pT7 Blue T-vector (Novagen) and recombinant clones in which single copies of the PCR fragment were inserted in either orientation were selected (pT71850-2360) and confirmed by restriction mapping. The insert was excised from two appropriately oriented independently isolated pT71850-2360 plasmids, with EcoRI (5' end of repeats) and PstI (in the flanking polylinker of the vector), and cloned into EcoRI/PstI cleaved pMalc vector. The resulting construct (pMB1850-2360) was confirmed by restriction analysis, and yielded 20 mg/l of soluble fusion protein [greater than 90% intact (i.e., nondegraded)] after affinity chromatography. Restriction of this plasmid with HindIII and religation of the vector resulted in the removal of the 1970-2360 interval. The resultant construct (pMB1850-1970) expressed greater than 70 mg/liter of 90% full length fusion protein.

Detailed Description Text (462):
The pPB1850-2360 construct was made by cloning a EcoRI (filled with Klenow)-BamHI fragment from a pT71850-2360 vector (opposite orientation to that used in the pMB1850-2360 construction) into NdeI (filled)/BamHI cleaved pET16b vector. Yields of affinity purified soluble fusion protein were 15 mg/liter, of greater than 90% full length fusion protein.

Detailed Description Text (466):
A second construct spanning this interval (aa 10-470) was constructed by cloning the PCR amplification product from a reaction containing the P9 (SEQ ID NO:15) and P10 (SEQ ID NO:16) primers (FIG. 18) into the pETHisa vector. This was accomplished by cloning the PCR product as an EcoRI-blunt fragment into EcoRI-HincII restricted vector DNA; recombinant clones were verified by restriction mapping. Although this construct (pPB 10-520) allowed expression and purification (utilizing the N-terminal polyhistidine affinity tag) of intact fusion protein, yields were estimated at less than 500 .mu.g per liter culture.

Detailed Description Text (467):
Higher yield of recombinant protein from this interval (aa 10-520) were obtained by expression of the interval in two overlapping clones. The 10-330aa interval was cloned in both pETHisa and pMalc vectors, using the BamHI-AflII (filled) DNA fragment from pPB10-520. This fragment was cloned into BamHI-HindIII (filled) restricted pMalc or BamHI-HincII restricted pETHisa vector. Recombinant clones were verified by restriction mapping. High level expression of either insoluble (pET) or soluble (pMal) fusion protein was obtained. Total yields of fusion protein from the pMB10-330 construct (FIG. 18) were 20 mg/liter culture, of which 10% was estimated to be full-length fusion protein. Although yields of this interval were higher and >90% full-length recombinant protein produced when expressed from the pET construct, the pMal fusion was utilized since the expressed protein was soluble and thus more likely to have the native conformation.

Detailed Description Text (468):
The pMB260-520 clone was constructed by cloning EcoRI-XbaI cleaved amplified DNA from a PCR reaction containing the P11 (SEQ ID NO:17) and P10 (SEQ ID NO:16) DNA primers (FIG. 18) into similarly restricted pMalc vector. Yields of affinity purified protein were 10 mg/liter, of which approximately 50% was estimated to be full-length recombinant protein.

Detailed Description Text (469):
The aa510-1110 interval was expressed as described below. This entire interval was expressed as a pMal fusion by cloning the NheI-HindIII fragment of pUCB10-1530 into XbaI-HindIII cleaved pMalc vector. The integrity of the construct (pMB510-1110) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. The yield of affinity purified protein was 25 mg/liter culture, of which 5% was estimated to be full-length fusion protein (1 mg/liter).

Detailed Description Text (471):
Recombinant protein expressed from the pMB510-820 vector was highly unstable. However, high levels (20 mg/liter) of >90% full-length fusion protein were obtained from the pMB820-1105 construct. The combination of partially degraded pMB510-1110 protein (enriched for the 510-820 interval) with the pMB820-1110 protein provides usable amounts of recombinant antigen from this interval.

Detailed Description Text (472):
The aa1100-1750 interval was expressed as described below. The entire interval was expressed in the pMalc vector from a construct in which the AccI(filled)-SpeI fragment of pPB10-1750 was inserted into StuI/XbaI (XbaI is compatible with SpeI; StuI and filled AccI sites are both blunt ended) restricted pMalc. The integrity of this construct (pMB1100-1750) was verified by restriction mapping and DNA sequencing of the clone junction using a MBP specific DNA primer. Although 15 mg/liter of affinity purified protein was isolated from cells harboring this construct, the protein was greater than 99% degraded to MBP monomer size.

Detailed Description Text (473):
A smaller derivative of pMB1100-1750 was constructed by restriction of pMB1100-1750 with AflII and SalI (in the pMalc polylinker 3' to the insert), filling in the overhanging ends, and religating the plasmid. The resultant clone (verified by restriction digestion and DNA sequencing) has deleted the aa1530-1750 interval, was designated pMB1100-1530. pMB1100-1530 expressed recombinant protein at a yield of greater than 40 mg/liter, of which 5% was estimated to be full-length fusion protein.

Detailed Description Text (474):
Three constructs were made to express the remaining interval. Initially, a BspHI (filled)-SpeI fragment from pPB10-1750 was cloned into EcoRI(filled)/XbaI cleaved pMalc vector. The integrity of this construct (pMB1570-1750) was verified by restriction

mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. Expression of recombinant protein from this plasmid was very low, approximately 3 mg affinity purified protein per liter, and most was degraded to MBP monomer size. This region was subsequently expressed from a PCR amplified DNA fragment. A PCR reaction utilizing primers P13 [SEQ ID NO: 18; P13 was engineered to introduce an EcoRI site 5' to amplified toxin B sequences] and P8 (SEQ ID NO:14) was performed on C. difficile genomic DNA as described above. The amplified fragment was cleaved with EcoRI and SpeI, and cloned into EcoRI/XbaI cleaved pMalc vector. The resultant clone (pMB1530-1750) was verified by restriction map analysis, and recombinant protein was expressed and purified. The yield was greater than 20 mg protein per liter culture and it was estimated that 25% was full-length fusion protein; this was a significantly higher yield than the original pMB1570-1750 clone. The insert of pMB1530-1750 (in a EcoR-SalI fragment) was transferred to the pETHisa vector (EcoRI/XhoI cleaved, XhoI and SalI ends are compatible). No detectable fusion protein was purified on Ni-Chelate columns from soluble lysates of cells induced to express fusion protein from this construct.

Detailed Description Text (483):
The above experiment was repeated, using two smaller expressed fragments (pMB 1750-1970 and pMB 1970-2360, see FIG. 19) comprising the INT-3 domain to determine if that domain could be further subdivided into smaller neutralizing epitopes. In addition, full-length INT-3 polypeptide expressed as a histidine tagged protein (pPB1750-2360) was tested for neutralizing ability and compared to the original INT-3 expressed MBP fusion (pMB1750-2360). The results are shown in Table 25.

Detailed Description Text (484):
The results summarized in Table 25 indicate that the smaller polypeptide fragments within the INT-3 domain, pMB1750-1970 and pMB1970-2360, partially lose the ability to bind to and remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that the full length INT-3 polypeptide is required to completely deplete the CTB antibody pool of neutralizing antibodies. This experiment also shows that the neutralization epitope of INT-3 can be expressed in alternative vector systems and the results are independent of the vector utilized or the accompanying fusion partner.

Detailed Description Text (490):
To determine if antibodies directed against the toxin B repeat region are sufficient for neutralization, region specific antibodies within the CTB antibody preparation were affinity purified, and tested for in vivo neutralization. Affinity colurines containing recombinant toxin B repeat proteins were made as described below. A separate affinity column was prepared using each of the following recombinant toxin B repeat proteins: pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB 1970-2360.

Detailed Description Text (491):
For each affinity column to be made, four ml of PBS-washed Actigel resin (Sterogene) was coupled overnight at room temperature with 5-10 mg of affinity purified recombinant protein (first extensively dialyzed into PBS) in 15 ml tubes (Falcon) containing a 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 30% coupling efficiencies were estimated. The resins were poured into 10 ml columns (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0) and reequilibrated in PBS. The columns were stored at 4.degree. C.

Detailed Description Text (492):
Aliquots of a CTB IgY polyclonal antibody preparation (PEG prep) were affinity purified on each of the four columns as described below. The columns were hooked to a UV monitor (ISCO), washed with PBS and 40 ml aliquots of a 2X PEG prep (filter sterilized using a 0.45.mu. filter) were applied. The columns were washed with PBS until the baseline value was re-established. The columns were then washed with BBStween to elute nonspecifically binding antibodies, and reequilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0). The eluted antibody was immediately dialyzed against a 100-fold excess of PBS at 4.degree. C. for 2 hrs. The samples were then dialyzed extensively against at least 2 changes of PBS, and affinity purified antibody was collected and stored at 4.degree. C. The antibody preparations were quantified by UV absorbance. The elution volumes were in the range of 4-8 ml. All affinity purified stocks contained similar total antibody concentrations, ranging from 0.25-0.35% of the total protein applied to the columns.

Detailed Description Text (493):

The ability of the affinity purified antibody preparations to neutralize toxin B in vivo was determined using the assay outlined in a) above. Affinity purified antibody was diluted 1:1 in PBS before testing. The results are shown in Table 26.

Detailed Description Text (494):
In all cases similar levels of toxin neutralization was observed, such that lethality was delayed in all groups relative to preimmune controls. This result demonstrates that antibodies reactive to the repeat region of the toxin B gene are sufficient to neutralize toxin B in vivo. The hamsters will eventually die in all groups, but this death is maximally delayed with the CTB PEG prep antibodies. Thus neutralization with the affinity purified (AP) antibodies is not as complete as that observed with the CTB prep before affinity chromatography. This result may be due to loss of activity during guanidine denaturation (during the elution of the antibodies from the affinity column) or the presence of antibodies specific to other regions of the toxin B gene that can contribute to toxin neutralization (present in the CTB PEG prep).

Detailed Description Text (495):
The observation that antibodies affinity purified against the non-overlapping pMB1750-1970 and pMB1970-2360 proteins neutralized toxin B raised the possibility that either 1) antibodies specific to repeat sub-regions are sufficient to neutralize toxin B or 2) sub-region specific proteins can bind most or all repeat specific antibodies present in the CTB polyclonal pool. This would likely be due to conformational similarities between repeats, since homology in the primary amino acid sequences between different repeats is in the range of only 25-75% [Eichel-Streiber, et al. (1992) Molec. Gen. Genetics 233:260]. These possibilities were tested by affinity chromatography.

Detailed Description Text (497):
As predicted by the affinity chromatography results, depletion of the CTB prep on the pMB1750-1970 column removed all detectable reactivity to the pMB1970-2360 protein. The reciprocal purification of a CTB prep that was depleted on the pMB1970-2360 column yielded no bound antibody when chromatographed on the pMB1750-1970 column. These results demonstrate that all repeat reactive antibodies in the CTB polyclonal pool recognize a conserved structure that is present in non-overlapping repeats. Although it is possible that this conserved structure represents rare conserved linear epitopes, it appears more likely that the neutralizing antibodies recognize a specific protein conformation. This conclusion was also suggested by the results of Western blot hybridization analysis of CTB reactivity to these recombinant proteins.

Detailed Description Text (500):
Although the CTB prep reacts with the pPB1750-2360, pPB1850-2360, and pMB 1970-2360 proteins, no reactivity to the pMB 1750-1970 protein was observed (FIG. 24). Given that all repeat reactive antibodies can be bound by this protein during affinity chromatography, this result indicates that the protein cannot fold properly on Western blots. Since this eliminates all antibody reactivity, it is unlikely that the repeat reactive antibodies in the CTB prep recognize linear epitopes. This may indicate that in order to induce protective antibodies, recombinant toxin B protein will need to be properly folded.

Detailed Description Text (508):
Affinity purification was utilized to determine if this difference in immunoreactivity by Western blot analysis reflects differing antibody titers. Fifty ml 2X PEG preparations from chickens immunized with either pMB1750-2360 or pMB1970-2360 protein were chromatographed on the pPB 1750-2360 affinity column from b) above, as described. The yield of affinity purified antibody (% total protein in preparation) was equivalent to the yield obtained from a CTB PEG preparation in b) above. Thus, differences in Western reactivity reflect a qualitative difference in the antibody pools, rather than quantitative differences., These results demonstrate that certain recombinant proteins are more effective at generating high affinity antibodies (as assayed by Western blot hybridization).

Detailed Description Text (511):
The ability of each immunogen to neutralize toxin B in vivo has been compiled and is shown in Table 30. As predicted from the recombinant protein-CTB premix studies (Table 24) only antibodies to Interval 3 (1750-2366) and not the other regions of toxin B (i.e., intervals 1-5) are protective. Unexpectedly, antibodies generated to INT-3 region expressed in pMAL vector (pMB1750-2360 and pMB1750-2360) using Freund's adjuvant were non-neutralizing. This observation is reproducible, since no neutralization was observed in two independent immunizations with pMB 1750-2360 and one immunization with

pMpB 1750-2360. The fact that 5X quantities of affinity purified toxin B repeat specific antibodies from pMB1750-2360 PEG preps cannot neutralize toxin B while 1X quantities of affinity purified anti-CTB antibodies can (Table 28) demonstrates that the differential ability of CTB antibodies to neutralize toxin B is due to qualitative rather than quantitative differences in these antibody preparations. Only when this region was expressed in an alternative vector (pPB1750-2360) or using an alternative adjuvant with the pMB1750-2360 protein were neutralizing antibodies generated. Importantly, antibodies raised using Freund's adjuvant to pPB 1850-2360, which contains a fragment that is only 100 amino acids smaller than recombinant pPB1750-2360, are unable to neutralize toxin B in vivo (Table 27); note also that the same vector is used for both pPB1850-2360 and pPB1750-2360.

Detailed Description Text (512):
The pPB1750-2360 antibody pool confers significant in vivo protection, equivalent to that obtained with the affinity purified CTB antibodies. This correlates with the observed high affinity of this antibody pool (relative to the pMB1750-2360 or pMB1970-2360 pools) as assayed by Western blot analysis (FIG. 24). These results provide the first demonstration that in vivo neutralizing antibodies can be induced using recombinant toxin B protein as immunogen.

Detailed Description Text (516):
In Example 19, it was demonstrated that toxin B neutralizing antibodies could be generated using specific recombinant toxin B proteins (pPB1750-2360) or specific adjuvants. Antibodies raised against pMB1750-2360 were capable of neutralizing the enterotoxic effect of toxin B when the recombinant protein was used to immunize hens in conjunction with the Gerbu adjuvant, but not when Freunds adjuvant was used. To determine the basis for these antigen and adjuvant restrictions, toxin B-specific antibodies present in the neutralizing and non-neutralizing PEG preparations were isolated by affinity chromatography and tested for qualitative or quantitative differences. The example involved a) purification of anti-toxin B specific antibodies from pMB1750-2360 and pPB1750-2360 PEG preparations and b) in vivo neutralization of toxin B using the affinity purified antibody.

Detailed Description Text (518):
To purify and determine the concentration of specific antibodies (expressed as the percent of total antibody) within the pPB1750-2360 (Freunds and Gerbu) and pPB1750-2360 PEG preparations, defined quantities of these antibody preparations were chromatographed on an affinity column containing the entire toxin B repeat region (pPB1750-2360). The amount of affinity purified antibody was then quantified.

Detailed Description Text (519):
An affinity column containing the recombinant toxin B repeat protein, pPB1750-2360, was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5 mg of pPB1750-2360 affinity purified protein (dialyzed into PBS; estimated to be greater than 95% full length fusion protein) in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, greater than 95% (approximately 5 mg) of recombinant protein was coupled to the resin. The coupled resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCL, pH 8.0; 0.005% thimerosal) and re-equilibrated in PBS and stored at 4.degree. C.

Detailed Description Text (520):
Aliquots of pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations (PEG preps) were affinity purified on the above column as follows. The column was attached to an UV monitor (ISCO), and washed with PBS. Forty ml aliquots of 2X PEG preps (filter sterilized using a 0.45.mu. filter and quantified by OD.sub.280 before chromatography) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCL, pH 8.0, 0.005% thimerosal) and the entire elution peak collected in a 15 ml tube (Falcon). The column was re-equilibrated, and the column eluate re-chromatographed as described above. The antibody preparations were quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Approximately 10 fold higher concentrations of total purified antibody was obtained upon elution of the first chromatography pass relative to the second pass. The low yield from the second chromatography pass indicated that most of the specific antibodies were removed by the first round of chromatography.

Detailed Description Text (521):
Pools of affinity purified specific antibodies were prepared by dialysis of the column elutes after the first column chromatography pass for the pMB1750-2360, pMB 1750-2360 (Gerbu) or pPB 1750-2360 IgY polyclonal antibody preparations. The elutes were collected on ice and immediately dialyzed against a 100-fold volume of PBS at 4.degree. C. for 2 hrs. The samples were then dialyzed against 3 changes of a 65-fold volume of PBS at 4.degree. C. Dialysis was performed for a minimum of 8 hrs per change of PBS. The dialyzed samples were collected, centrifuged to remove insoluble debris, quantified by OD.sub.280, and stored at 4.degree. C.

Detailed Description Text (522):
The percentage of toxin B repeat-specific antibodies present in each preparation was determined using the quantifications of antibody yields from the first column pass (amount of specific antibody recovered after first pass/total protein loaded). The yield of repeat-specific affinity purified antibody (expressed as the percent of total protein in the preparation) in: 1) the pMB1750-2360 PEG prep was approximately 0.5%, 2) the pMB1750-2360 (Gerbu) prep was approximately 2.3%, and 3) the pPB1750-2360 prep was approximately 0.4%. Purification of a CTB IgY polyclonal antibody preparation on the same column demonstrated that the concentration of toxin B repeat specific antibodies in the CTB preparation was 0.35%.

Detailed Description Text (523):
These results demonstrate that 1) the use of Gerbu adjuvant enhanced the titer of specific antibody produced against the pMB1750-2360 protein 5-fold relative to immunization using Freunds adjuvant, and 2) the differences seen in the in vivo neutralization ability of the pMB 1750-2360 (not neutralizing) and pPB 1750-2360 (neutralizing) and CTB (neutralizing) PEG preps seen in Example 19 was not due to differences in the titers of repeat-specific antibodies in the three preparations because the titer of repeat-specific antibody was similar for all three preps; therefore the differing ability of the three antibody preparations to neutralize toxin B must reflect qualitative differences in the induced toxin B repeat-specific antibodies. To confirm that qualitative differences exist between antibodies raised in hens immunized with different recombinant proteins and/or different adjuvants, the same amount of affinity purified anti-toxin B repeat (aa 1870-2360 of toxin B) antibodies from the different preparations was administered to hamsters using the in vivo hamster model as described below.

Detailed Description Text (524):
b) In vivo Neutralization Of Toxin B Using Affinity Purified Antibody

Detailed Description Text (525):
The in vivo hamster model was utilized to assess the neutralizing ability of the affinity purified antibodies raised against recombinant toxin B proteins purified in (a) above. As well, a 4X IgY PEG preparation from a second independent immunization utilizing the pPB1750-2360 antigen with Freunds adjuvant was tested for in vivo neutralization. The results are shown in Table 31.

Detailed Description Text (527):
1) as shown in Example 19 and reproduced here, 1.5 mg of affinity purified antibody from pMB1750-2360 immunized hens using Freunds adjuvant does not neutralize toxin B in vivo. However, 300 .mu.g of affinity purified antibody from similarly immunized hens utilizing Gerbu adjuvant demonstrated complete neutralization of toxin B in vivo. This demonstrates that Gerbu adjuvant, in addition to enhancing the titer of antibodies reactive to the pMB1750-2360 antigen relative to Freunds adjuvant (demonstrated in (a) above), also enhances the yield of neutralizing antibodies to this antigen, greater than 5 fold.

Detailed Description Text (528):
2) Complete in vivo neutralization of toxin B was observed with 1.5 mg of affinity purified antibody from hens immunized with pPB1750-2360

Detailed Description Text (531):
4) Complete neutralization of toxin B was observed using 300 .mu.g of CTB antibody [affinity purified (AP)] but not 100 .mu.g CTB antibody (AP or PEG prep). This demonstrates that greater than 100 .mu.g of toxin B repeat-specific antibody (anti-CTB) is necessary to neutralize 25 .mu.g toxin B in vivo in this assay, and that affinity purified antibodies specific to the toxin B repeat interval neutralize toxin B as effectively as the PEP prep of IgY raised against the entire CTB protein (shown in this

assay).

Detailed Description Text (535):
The ability of the recombinant toxin proteins and antibodies raised against these recombinant proteins (described in the above examples) to form the basis of diagnostic assays for the detection of clostridial toxin in a sample was examined. Two immunoassay formats were tested to quantitatively detect C. difficile toxin A and toxin B from a biological specimen. The first format involved a competitive assay in which a fixed amount of recombinant toxin A or B was immobilized on a solid support (e.g., microtiter plate wells) followed by the addition of a toxin-containing biological specimen mixed with affinity-purified or PEG fractionated antibodies against recombinant toxin A or B. If toxin is present in a specimen, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of a reporter reagent. The reporter reagent detects the presence of antibody bound to the immobilized toxin protein.

Detailed Description Text (536):
In the second format, a sandwich immunoassay was developed using affinity-purified antibodies to recombinant toxin A and B. The affinity-purified antibodies to recombinant toxin A and B were used to coat microtiter wells instead of the recombinant polypeptides (as was done in the competitive assay format). Biological samples containing toxin A or B were then added to the wells followed by the addition of a reporter reagent to detect the presence of bound toxin in the well.

Detailed Description Text (538):
Recombinant toxin A or B was attached to a solid support by coating 96 well microtiter plates with the toxin protein at a concentration of 1 .mu.g/ml in PBS. The plates were incubated overnight at 2.degree.-8.degree. C. The following morning, the coating solutions were removed and the remaining protein binding sites on the wells were blocked by filling each well with a PBS solution containing 0.5% BSA and 0.05% Tween-20. Native C. difficile toxin A or B (Tech Lab) was diluted to 4 .mu.g/ml in stool extracts from healthy Syrian hamsters (Sasco). The stool extracts were made by placing fecal pellets in a 15 ml centrifuge tube; PBS was added at 2 ml/pellet and the tube was vortexed to create a uniform suspension. The tube was then centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was removed; this comprises the stool extract. Fifty .mu.l of the hamster stool extract was pipetted into each well of the microtiter plates to serve as the diluent for serial dilutions of the 4 .mu.g/ml toxin samples. One hundred .mu.l of the toxin samples at 4 .mu.g/ml was pipetted into the first row of wells in the microtiter plate, and 50 .mu.l aliquots were removed and diluted serially down the plate in duplicate. An equal volume of affinity purified anti-recombinant toxin antibodies [1 ng/well of anti-pMA1870-2680 antibody was used for the detection of toxin A; 0.5 ng/well of anti-pMB1750-2360(Gerbu) was used for the detection of toxin B] were added to appropriate wells, and the plates were incubated at room temperature for 2 hours with gentle agitation. Wells serving as negative control contained antibody but no native toxin to compete for binding.

Detailed Description Text (540):
The results are summarized in Tables 32 and 33. For the results shown in Table 32, the wells were coated with recombinant toxin A protein (pMA1870-2680). The amount of native toxin A added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin A protein, pMA1870-2680, was affinity purified on the an affinity column containing pPA1870-2680 (described in Example 20). As shown in Table 32, the recombinant toxin A protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin A in biological samples.

Detailed Description Text (541):
Similar results were obtained using the recombinant toxin B, pPB1750-2360, and antibodies raised against pMB1750-2360(Gerbu). For the results shown in Table 33, the wells were coated with recombinant toxin B protein (pPB1750-2360). The amount of native toxin B added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin B protein, pMB1750-2360(Gerbu), was affinity purified on the an affinity column containing pPB1850-2360 (described in Example 20). As shown in Table 33, the recombinant toxin B protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin B in biological samples.

Detailed Description Text (545):
Affinity-purified antibodies against recombinant toxin A or toxin B were immobilized to

96 well microtiter plates as follows. The wells were passively coated overnight at 4.degree. C. with affinity purified antibodies raised against either pMA1870-2680 (toxin A) or pMB1750-2360(Gerbu) (toxin B). The antibodies were affinity purified as described in Example 20. The antibodies were used at a concentration of 1 .mu.g/ml and 100 .mu.l was added to each microtiter well. The wells were then blocked with 200 .mu.l of 0.5% BSA in PBS for 2 hours at room temperature and the blocking solution was then decanted. Stool samples from healthy Syrian hamsters were resuspended in PBS, pH 7.4 (2 ml PBS/stool pellet was used to resuspend the pellets and the sample was centrifuged as described above). The stool suspension was then spiked with native C. difficile toxin A or B (Tech Lab) at 4 .mu.g/ml. The stool suspensions containing toxin (either toxin A or toxin B) were then serially diluted two-fold in stool suspension without toxin and 50 .mu.l was added in duplicate to the coated microtiter wells. Wells containing stool suspension without toxin served as the negative control.

Detailed Description Text (550):
Construction And Expression Of C. botulinum C Fragment Fusion Proteins

Detailed Description Text (551):
The C. botulinum type A neurotoxin gene has been cloned and sequenced [Thompson, et al., Eur. J. Biochem. 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066; the nucleotide sequence of the coding region is listed in SEQ ID NO:27. The amino acid sequence of the C. botulinum type A neurotoxin is listed in SEQ ID NO:28. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H.sub.c domain.

Detailed Description Text (552):
Previous attempts by others to express polypeptides comprising the C fragment of C. botulinum type A toxin as a native polypeptide (e.g., not as a fusion protein) in E. coli have been unsuccessful [H. F. LaPenotiere, et al. in Botulinum and Tetanus Neurotoxins, DasGupta, Ed., Plenum Press, New York (1993), pp. 463-466]. Expression of the C fragment as a fusion with the E. coli MBP was reported to result in the production of insoluble protein (H. F. LaPenotiere, et al., supra).

Detailed Description Text (553):
In order to produce soluble recombinant C fragment proteins in E. coli, fusion proteins comprising a synthetic C fragment gene derived from the C. botulinum type A toxin and either a portion of the C. difficile toxin protein or the MBP were constructed. This example involved a) the construction of plasmids encoding C fragment fusion proteins and b) expression of C. botulinum C fragment fusion proteins in E. coli.

Detailed Description Text (554):
a) Construction Of Plasmids Encoding C Fragment Fusion Proteins

Detailed Description Text (555):
In Example 11, it was demonstrated that the C. difficile toxin A repeat domain can be efficiently expressed and purified in E. coli as either native (expressed in the pET 23a vector in clone pPA1870-2680) or fusion (expressed in the pMALc vector as a fusion with the E. coli MBP in clone pMA1870-2680) proteins. Fusion proteins comprising a fusion between the MBP, portions of the C. difficile toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the C. botulinum type A toxin were constructed. A fusion protein comprising the C fragment of the C. botulinum type A toxin and the MBP was also constructed.

Detailed Description Text (556):
FIG. 25 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the C. difficile toxin A sequences or C. botulinum C fragment sequences which were used to generate the botulinal fusion proteins. In FIG. 25, the solid boxes represent C. difficile toxin A gene sequences, the open boxes represent C. botulinum C fragment sequences and the solid black ovals represent the E. coli MBP. When the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

Detailed Description Text (557):
In FIG. 25, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs

(described in Example 11) which contain sequences derived from the C. difficile toxin A repeat domain are shown; these constructs were used as the source of C. difficile toxin A gene sequences for the construction of plasmids encoding fusions between the C. botulinum C fragment gene and the C. difficile toxin A gene. The pMA1870-2680 expression construct expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column (purification described in Example 11d).

Detailed Description Text (558):
The pAlterBot construct (FIG. 25) was used as the source of C. botulinum C fragment gene sequences for the botulinal fusion proteins. pAlterBot was obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense. pAlterBot contains a synthetic C. botulinum C fragment inserted in to the pALTER-1.RTM. vector (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson et al., supra). This high A/T content creates expression difficulties in E. coli and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to improve the expression of C fragment proteins in E. coli, a synthetic version of the gene was created in which the non-preferred codons were replaced with preferred codons.

Detailed Description Text (559):
The nucleotide sequence of the C. botulinum C fragment gene sequences contained within pAlterBot is listed in SEQ ID NO:22. The first six nucleotides (ATGGCT) encode a methionine and alanine residue, respectively. These two amino acids result from the insertion of the C. botulinum C fragment sequences into the pALTER.RTM. vector and provide the initiator methionine residue. The amino acid sequence of the C. botulinum C fragment encoded by the sequences contained within pAlterBot is listed in SEQ ID NO:23. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the C. botulinum type A toxin gene.

Detailed Description Text (560):
The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used as progenitor plasmids to make expression constructs in which fragments of the C. difficile toxin A repeat domain were expressed as genetic fusions with the C. botulinum C fragment gene using the pMAL-c expression vector (New England BioLabs). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the C. botulinum C fragment gene was expressed as a fusion with only the MBP was constructed (FIG. 25). Fusion protein expression was induced from E. coli strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

Detailed Description Text (562):
In order to facilitate the cloning of the C. botulinum C fragment gene sequences into a number of desired constructs, the botulinal gene sequences were removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (FIG. 25). pBlueBot was constructed as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and plasmid DNA was isolated using the QIAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with NcoI and the resulting 3' recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with HindIII to release the botulinal gene sequences (the Bot insert) as a blunt (filled NcoI site)-HindIII fragment. pBluescript vector DNA was prepared by digesting 200 ng of pBluescript DNA with SmaI and HindIII. The digestion products from both plasmids were resolved on an agarose gel. The appropriate fragments were removed from the gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was then ligated using T4 DNA ligase and used to transform competent DH5.alpha. cells (Gibco-BRL). Host cells were made competent for transformation using the calcium chloride protocol of Sambrook et al., supra at 1.82-1.83. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook et al, supra). The resultant clone, pBlueBot, contains several useful unique restriction sites flanking the Bot insert (i.e., the C. botulinum C fragment sequences derived from pAlterBot) as shown in FIG. 25.

Detailed Description Text (563):
ii) Construction Of C. difficile/C. botulinum /MBP Fusion Proteins

Detailed Description Text (564):

Constructs encoding fusions between the C. difficile toxin A gene and the C. botulinum C fragment gene and the MBP were made utilizing the same recombinant DNA methodology outlined above; these fusion proteins contained varying amounts of the C. difficile toxin A repeat domain.

Detailed Description Text (565):
The pMABot clone contains a 2.4 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e., the C. botulinum C fragment sequences derived from pAlterBot). pMABot (FIG. 25) was constructed by mixing gel-purified DNA from NotI/HindIII digested pBlueBot (the 1.2 kb Bot fragment), SpeI/NotI digested pPA1100-2680 (the 2.4 kb C. difficile toxin A repeat fragment) and XbaI/HindIII digested pMAL-c vector. Recombinant clones were isolated, confirmed by restriction digestion and purified using the QIAprep-spin Plasmid Kit (Qiagen). This clone expresses the toxin A repeats and the botulinal C fragment protein sequences as an in-frame fusion with the MBP.

Detailed Description Text (566):
The pMCABot construct contains a 1.0 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e, the C. botulinum C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with EcoRI to remove the 5' end of the C. difficile toxin A repeat (see FIG. 25, the pMAL-c vector contains a EcoRI site 5' to the C. difficile insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot, FIG. 25) generated an in-frame fusion between the MBP and the remaining 3' portion of the C. difficile toxin A repeat domain fused to the Bot gene.

Detailed Description Text (567):
The pMNABot clone contains the 1 kb SpeI/EcoRI (filled) fragment from the C. difficile toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb C. botulinum C fragment gene as a NcoI (filled)/HindIII fragment (derived from pAlterBot). These two fragments were inserted into the pMAL-c vector digested with XbaI/HindIII. The two insert fragments were generated by digestion of the appropriate plasmid with EcoRI (pPA1100-2680) or NcoI (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either SpeI or HindIII). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis; the EcoRI site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled EcoRI and NcoI sites.

Detailed Description Text (568):
A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (i.e., this fusion lacks any C. difficile toxin A gene sequences) and termed pMBot. The pMBot construct was made by removal of the C. difficile toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This was accomplished by digestion of pMABot DNA with StuI (located in the pMALc polylinker 5' to the XbaI site) and XbaI (located 3' to the NotI site at the toxA-Bot fusion junction), filling in the XbaI site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (i.e, the C. botulinum C fragment sequences).

Detailed Description Text (569):
b) Expression Of C. botulinum C Fragment Fusion Proteins In E. coli

Detailed Description Text (570):
Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) were grown, induced, and soluble protein fractions were isolated as described in Example 18. The soluble protein extracts were chromatographed on amylose affinity columns to isolate recombinant fusion protein. The purified recombinant fusion proteins were analyzed by running samples on SDS-PAGE gels followed by Coomassie staining and by Western blot analysis as described [Williams et al, (1994) supra]. In brief, extracts were prepared and chromatographed in column buffer (10 mM NaPO.sub.4, 0.5M NaCl, 10 mM .beta.-mercaptoethanol, pH 7.2) over an amylose resin (New England Biolabs) column, and eluted with column buffer containing 10 mM maltose as described [Williams, et al. (1994), supra]. An SDS-PAGE gel containing the purified protein samples stained with Coomassie blue is shown in FIG. 26.

Detailed Description Text (572):

The protein samples were prepared for electrophoresis by mixing 5 .mu.l of eluted protein with 5 .mu.l of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; .beta.-mercaptoethanol is added to 5% before use). The samples were heated to 95.degree. C. for 5 min, then cooled and loaded on a 7.5% agarose SDS-PAGE gel. Broad range molecular weight protein markers were also loaded to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected generally by staining the gel with Coomassie blue.

Detailed Description Text (573):
In all cases the yields were in excess of 20 mg fusion protein per liter culture (see Table 36) and, with the exception of the pMCABot protein, a high percentage (i.e., greater than 20-50% of total eluted protein) of the eluted fusion protein was of a MW predicted for the full length fusion protein (FIG. 26). It was estimated (by visual inspection) that less than 10% of the pMCABot fusion protein was expressed as the full length fusion protein.

Detailed Description Text (574):
These results demonstrate that high level expression of intact C. botulinum C fragment/C. difficile toxin A fusion proteins in E. coli is feasible using the pMAL-c expression system. These results are in contrast to those reported by H. F. LaPenotiere, et al. (1993), supra. In addition, these results show that it is not necessary to fuse the botulinal C fragment gene to the C. difficile toxin A gene in order to produce a soluble fusion protein using the pMAL-c system in E. coli.

Detailed Description Text (575):
In order to determine whether the above-described botulinal fusion proteins were recognized by anti-C. botulinum toxin A antibodies, Western blots were performed. Samples containing affinity-purified proteins from E. coli containing the pMABot, pMCABot, pMNABot, pMBot, pMA1870-2680 or pMALc plasmids were analyzed. SDS-PAGE gels (7.5% acrylamide) were loaded with protein samples purified from each expression construct. After electrophoresis, the gels were blotted and protein transfer was confirmed by Ponceau S staining (as described in Example 12b).

Detailed Description Text (576):
Following protein transfer, the blots were blocked by incubation for 1 hr at 20.degree. C. in blocking buffer [PBST (PBS containing 0.1% Tween 20 and 5% dry milk)]. The blots were then incubated in 10 ml of a solution containing the primary antibody; this solution comprised a 1/500 dilution of an anti-C. botulinum toxin A IgY PEG prep (described in Example 3) in blocking buffer. The blots were incubated for 1 hr at room temperature in the presence of the primary antibody. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the secondary antibody as follows. The rabbit anti-chicken antibody was diluted to 1 .mu.g/ml in blocking buffer (10 ml final volume per blot) and the blots were incubated at room temperature for 1 hour in the presence of the secondary antibody. The blots were then washed successively with PBST, BBS-Tween and 50 mM Na.sub.2 CO.sub.3, pH 9.5. The blots were then developed in freshly-prepared alkaline phosphatase substrate buffer (100 .mu.g/ml nitro blue tetrazolium, 50 .mu.g/ml 5-bromo-chloro-indolylphosphate, 5 mM MgCl.sub.2 in 50 mM Na.sub.2 CO.sub.3, pH 9.5). Development was stopped by flooding the blots with distilled water and the blots were air dried.

Detailed Description Text (577):
This Western blot analysis detected anti-C. botulinum toxin reactive proteins in the pMABot, pMCABot, pMNABot and pMBot protein samples (corresponding to the predicted full length proteins identified above by Coomassie staining in FIG. 26), but not in the pMA1100-2680 or pMALc protein samples.

Detailed Description Text (578):
These results demonstrate that the relevant fusion proteins purified on an amylose resin as described above in section a) contained immunoreactive C. botulinum C fragment protein as predicted.

Detailed Description Text (581):
The ability of the recombinant botulinal toxin proteins produced in Example 22 to stimulate a systemic immune response against botulinal toxin epitopes was assessed. This example involved: a) the evaluation of the induction of serum IgG titers produced by nasal or oral administration of botulinal toxin-containing C. difficile toxin A fusion proteins and b) the in vivo neutralization of C. botulinum type A neurotoxin by

anti- recombinant C. botulinum C fragment antibodies.

Detailed Description Text (582):
a) Evaluation Of The Induction Of Serum IgG Titers Produced By Nasal Or Oral Administration Of Botulinal Toxin-Containing C. difficile Toxin A Fusion Proteins

Detailed Description Text (584):
The fusion proteins were prepared for immunization as follows. The proteins (in column buffer containing 10 mM maltose) were diluted in 0.1M carbonate buffer, pH 9.5 and administered orally or nasally in a 200 .mu.l volume. The rats were lightly sedated with ether prior to administration. The oral dosing was accomplished using a 20 gauge feeding needle. The nasal dosing was performed using a P-200 micro-pipettor (Gilson). The rats were boosted 14 days after the primary immunization using the techniques described above and were bled 7 days later. Rats from each group were lightly etherized and bled from the tail. The blood was allowed to clot at 37.degree. C. for 1 hr and the serum was collected.

Detailed Description Text (585):
The serum from individual rats was analyzed using an ELISA to determine the anti-C. botulinum type A toxin IgG serum titer. The ELISA protocol used is a modification of that described in Example 13c. Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with C. botulinum type A toxoid (prepared as described in Example 3a) by placing 100 .mu.l volumes of C. botulinum type A toxoid at 2.5 .mu.g/ml in PBS containing 0.005% thimerosal in each well and incubating overnight at 4.degree. C. The next morning, the coating suspensions were decanted and all wells were washed three times using PBS.

Detailed Description Text (587):
The above ELISA results demonstrate that reactivity against the botulinal fusion proteins was strongest when the route of administration was nasal; only weak responses were stimulated when the botulinal fusion proteins were given orally. Nasally delivered pMbot and pMBot admixed with pMA1870-2680 invoked the greatest serum IgG response. These results show that only the pMBot protein is necessary to induce this response, since the addition of the pMA1870-2680 protein did not enhance antibody response (Table 37). Placement of the C. difficile toxin A fragment between the MBP and the C. botulinum C fragment protein dramatically reduced anti-bot IgG titer (see results using pMABot, pMCABot and pMNABot proteins).

Detailed Description Text (588):
This study demonstrates that the pMBot protein induces a strong serum IgG response directed against C. botulinum type A toxin when nasally administered.

Detailed Description Text (589):
b) In Vivo Neutralization Of C. botulinum Type A Neurotoxin By Anti- Recombinant C. botulinum C Fragment Antibodies

Detailed Description Text (590):
The ability of the anti-C. botulinum type A toxin antibodies generated by nasal administration of recombinant botulinal fusion proteins in rats (Example 22) to neutralize C. botulinum type A toxin was tested in a mouse neutralization model. The mouse model is the art accepted method for detection of botulinal toxins in body fluids and for the evaluation of anti-botulinal antibodies [E. J. Schantz and D. A. Kautter, J. Assoc. Off. Anal. Chem. 61:96 (1990) and Investigational New Drug (BB-IND-3703) application by the Surgeon General of the Department of the Army to the Federal Food and Drug Administration]. The anti-C. botulinum type A toxin antibodies were prepared as follows.

Detailed Description Text (591):
Rats from the group given pMBot protein by nasal administration were boosted a second time with 250 .mu.g pMBot protein per rat and serum was collected 7 days later. Serum from one rat from this group and from a preimmune rat was tested for anti-C. botulinum type A toxin neutralizing activity in the mouse neutralization model described below.

Detailed Description Text (592):
The LD.sub.50 of a solution of purified C. botulinum type A toxin complex, obtained from Dr. Eric Johnson (University of Wisconsin Madison), was determined using the intraperitoneal (IP) method of Schantz and Kautter [J. Assoc. Off. Anal. Chem. 61:96 (1978)] using 18-22 gram female ICR mice and was found to be 3500 LD.sub.50 /ml. The determination of the LD.sub.50 was performed as follows. A Type A toxin standard was

prepared by dissolving purified type A toxin complex in 25 mM sodium phosphate buffer, pH 6.8 to yield a stock toxin solution of 3.15.times.10.sup.7 LD.sub.50 /mg. The OD.sub.278 of the solution was determined and the concentration was adjusted to 10-20 .mu.g/ml. The toxin solution was then diluted 1:100 in gel-phosphate (30 mM phosphate, pH 6.4; 0.2% gelatin). Further dilutions of the toxin solution were made as shown below in Table 39. Two mice were injected IP with 0.5 ml of each dilution shown and the mice were observed for symptoms of botulism for a period of 72 hours.

Detailed Description Text (596):
As shown in Table 40 pMBot serum neutralized C. botulinum type A toxin complex when used at a dilution of 1:320 or less. A mean neutralizing value of 168 IU/ml was obtained for the pMBot serum (an IU is defined as 10,000 mouse LD.sub.50). This value translates to a circulating serum titer of about 3.7 IU/mg of serum protein. This neutralizing titer is comparable to the commercially available bottled concentrated (Connaught Laboratories, Ltd.) horse anti-C. botulinum antiserum. A 10 ml vial of Connaught antiserum contains about 200 mg/ml of protein; each ml can neutralize 750

Detailed Description Text (597):
IU of C. botulinum type A toxin. After administration of one vial to a human, the circulating serum titer of the Connaught preparation would be approximately 25 IU/ml assuming an average serum volume of 3 liters). Thus, the circulating anti-C. botulinum titer seen in rats nasally immunized with pMBot protein (168 IU/ml) is 6.7 time higher than the necessary circulation titer of anti-C. botulinum antibody needed to be protective in humans.

Detailed Description Text (598):
These results demonstrate that antibodies capable of neutralizing C. botulinum type A toxin are induced when recombinant C. botulinum C fragment fusion protein produced in E. coli is used as an immunogen.

Detailed Description Text (600):
Production Of Soluble C. botulinum C Fragment Protein Substantially Free Of Endotoxin Contamination

Detailed Description Text (601):
Example 23 demonstrated that neutralizing antibodies are generated by immunization with the pMBot protein expressed in E. coli. These results showed that the pMBot fusion protein is a good vaccine candidate. However, immunogens suitable for use as vaccines should be pyrogen-free in addition to having the capability of inducing neutralizing antibodies. Expression clones and conditions that facilitate the production of C. botulinum C fragment protein for utilization as a vaccine were developed.

Detailed Description Text (602):
The example involved: (a) determination of pyrogen content of the pMBot protein; (b) generation of C. botulinum C fragment protein free of the MBP; (c) expression of C. botulinum C fragment protein using various expression vectors; and (d) purification of soluble C. botulinum C fragment protein substantially free of significant endotoxin contamination.

Detailed Description Text (604):
In order to use a recombinant antigen as a vaccine in humans or other animals, the antigen preparation must be shown to be free of pyrogens. The most significant pyrogen present in preparations of recombinant proteins produced in gram-negative bacteria, such as E. coli, is endotoxin [F. C. Pearson, Pyrogens: endotoxins, LAL testing and depyrogentaion, (1985) Marcel Dekker, New York, pp. 23-56]. To evaluate the utility of the pMBot protein as a vaccine candidate, the endotoxin content in MBP fusion proteins was determined.

Detailed Description Text (605):
The endotoxin content of recombinant protein samples was assayed utilizing the Limulus assay (LAL kit; Associates of Cape Cod) according to the manufacturer's instructions. Samples of affinity-purified pMal-c protein and pMA1870-2680 were found to contain high levels of endotoxin [>50,000 EU/mg protein; EU (endotoxin unit)]. This suggested that MBP- or toxin A repeat-containing fusions with the botulinal C fragment should also contain high levels of endotoxin. Accordingly, removal of endotoxin from affinity-purified pMal-c and pMBot protein preparations was attempted as follows.

Detailed Description Text (607):
The depyrogenation experiment was repeated using an independently purified pMal-c

protein preparation and similar results were obtained. From these studies it was concluded that significant levels of endotoxin copurifies with these MBP fusion proteins using the amylose resin. Furthermore, this endotoxin cannot be easily removed by polymyxin treatment.

Detailed Description Text (608):
These results suggest that the presence of the MBP sequences on the fusion protein complicated the removal of endotoxin from preparations of the pMBot protein.

Detailed Description Text (609):
b) Generation Of C. botulinum C Fragment Protein Free Of The MBP

Detailed Description Text (610):
It was demonstrated that the pMBot fusion protein could not be easily purified from contaminating endotoxin in section a) above. The ability to produce a pyrogen-free (e.g., endotoxin-free) preparation of soluble botulinal C fragment protein free of the MBP tag was next investigated. The pMBot expression construct was designed to facilitate purification of the botulinal C fragment from the MBP tag by cleavage of the fusion protein by utilizing an engineered Factor Xa cleavage site present between the MBP and the botulinal C fragment. The Factor Xa cleavage was performed as follows.

Detailed Description Text (613):
1) Most (about 90%) pMBot protein could be removed by centrifugation, even when uncleaved control samples were utilized. This indicated that the pMBot fusion protein was not fully soluble (i.e., it exists as a suspension rather than as a solution). [This result was consistent with the observation that most affinity-purified pMBot protein precipitates after long term storage (>2 weeks) at 4.degree. C. Additionally, the majority (i.e., 75%) of induced pMBot protein remains in the pellet after sonication and clarification of the induced E. coli. Resuspension of these insoluble pellets in PBS followed by sonication results in partial solubilization of the insoluble pMBot protein in the pellets.]

Detailed Description Text (616):
4) The use of 0.1% SDS in the buffer used for Factor Xa cleavage enhanced the solubility of the pMBot protein (all of pMBot protein was soluble). However, the presence of the SDS prevented any cleavage of the fusion protein with Factor Xa.

Detailed Description Text (618):
These results demonstrate that purification of soluble botulinal C fragment protein after cleavage of the pMBot fusion protein is complicated by the insolubility of both the pMBot protein and the cleaved botulinal C fragment protein.

Detailed Description Text (619):
c) Expression Of C. botulinum C Fragment Using Various Expression Vectors

Detailed Description Text (620):
In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids were constructed. These expression constructs were generated utilizing the methodologies described in Example 22. FIG. 27 provides a schematic representation of the vectors described below.

Detailed Description Text (621):
In FIG. 27, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX3T vector (described in Example 11). The solid black lines represent C. botulinum C fragment gene sequences; the solid black ovals represent the MBP; the hatched ovals represent GST; "HHHHH" (SEQ ID NO: 32) represents the poly-histidine tag. In FIG. 27, when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

Detailed Description Text (623):
In order to express the C. botulinum C fragment as a native (i.e., non-fused) protein, the pPBot plasmid (shown schematically in FIG. 27) was constructed as follows. The C fragment sequences present in pAlterBot (Example 22) were removed by digestion of pAlterBot with NcoI and HindIII. The NcoI/HindIII C fragment insert was ligated to

pETHisa vector (described in Example 18b) which was digested with NcoI and HindIII. This ligation creates an expression construct in which the NcoI-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping.

Detailed Description Text (625):
In order to express the C. botulinum C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in FIG. 27) was constructed as follows. The NcoI/HindIII botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with NheI and HindIII. The NcoI (on the C fragment insert) and NheI (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the NdeI site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping.

Detailed Description Text (626):
The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHislleGluGlyArgHisMetAla, (SEQ ID NO:24); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is listed in SEQ ID NO:25. The amino acid sequence of the pHisBot protein is listed in SEQ ID NO:26.

Detailed Description Text (628):
The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in FIG. 27). This expression construct was created by cloning the NotI/SalI C fragment insert present in pBlueBot (Example 22) into the pGEX3T vector which was digested with Sinai and XhoI. The NotI site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells.

Detailed Description Text (630):
Large scale (1 liter) cultures of pPBot [BL21(DE3)pLysS host], pHisBot [BL21(DE3)pLysS host] and pGBot (BL21 host) were grown in 2X YT medium and induced (using IPTG to 0.8-1.0 mM) for 3 hrs as described in Example 22. Total, soluble and insoluble protein preparations were prepared from 1 ml aliquots of each large scale culture [Williams et al. (1994), supra] and analyzed by SDS-PAGE. No obvious induced band was detectable in the pPBot or pHisBot samples by Coomassie staining, while a prominent insoluble band of the anticipated MW was detected in the pGBot sample. Soluble lysates of the pGBot large scale (resuspended in PBS) or pHisBot large scale [resuspended in Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9)] cultures were prepared and used to affinity purify soluble affinity-tagged protein as follows.

Detailed Description Text (631):
The pGBot lysate was affinity purified on a glutathione-agarose resin (Pharmacia) exactly as described in Smith and Corcoran [Current Protocols in Molecular Biology, Supplement 28 (1994), pp. 16.7.1-16.7.7]. The pHisBot protein was purified on the His-Bind resin (Novagen) utilizing the His-bind buffer kit (Novagen) exactly as described by manufacturer.

Detailed Description Text (632):
Samples from the purification of both the pGBot and pHisBot proteins (including uninduced, induced, total, soluble, and affinity-purified eluted protein) were resolved on SDS-PAGE gels. Following electrophoresis, proteins were analyzed by Coomassie staining or by Western blot detection utilizing a chicken anti-C. botulinum Type A toxoid antibody (as described in Example 22).

Detailed Description Text (633):
These studies showed that the pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. Affinity purification of the pHisBot protein on this first attempt was inefficient, both in terms of yield (most of the immunoreactive botulinal protein did not bind to the His-bind resin) and purity (the botulinal protein was estimated to comprise approximately 20% of the total eluted protein).

Detailed Description Text (634):
d) Purification Of Soluble C. botulinum C Fragment Protein Substantially Free Of Endotoxin Contamination

Detailed Description Text (635):
The above studies showed that the pHisBot protein was expressed in E. coli as a soluble protein. However, the affinity purification of this protein on the His-bind resin was very inefficient. In order to improve the affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin; Qiagen) was utilized. The Ni-NTA resin was reported to have a superior binding affinity (K.sub.d =1.times.10.sup.-13 at pH 8.0; Qiagen user manual) relative to the His-bind resin.

Detailed Description Text (638):
Samples of total, soluble and eluted protein were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis as described in Example 22b. Duplicate gels were stained with Coomassie blue to visualize the resolved proteins and C. botulinum type A toxin-reactive protein was detected by Western blot analysis as described in Example 22b. A representative Coomassie stained gel is shown in FIG. 28. In FIG. 28, the following samples were loaded on the 12.5% acrylamide gel. Lanes 1-4 contain respectively total protein, soluble protein, soluble protein present in the flow-through of the Ni-NTA column and affinity-purified pHisBot protein (i.e., protein released from the Ni-NTA resin by protonation). Lane 5 contains high molecular weight protein markers (BioRad).

Detailed Description Text (639):
The purification of pHisBot protein resulted in a yield of 7 mg of affinity purified protein from a 1 liter starting culture of BL21(DE3)pLysS cells harboring the pHisBot plasmid. The yield of purified pHisBot protein represented approximately 0.4% of the total soluble protein in the induced culture. Analysis of the purified pHisBot protein by SDS-PAGE revealed that at least 90-95% of the protein was present as a single band (FIG. 28) of the predicted MW (50 kD). This 50 kD protein band was immunoreactive with anti-C. botulinum type A toxin antibodies. The extinction coefficient of the protein preparation was determined to be 1.4 (using the Pierce BCA assay) or 1.45 (using the Lowry assay) OD.sub.280 per 1 mg/ml solution.

Detailed Description Text (641):
Determination of endotoxin contamination in the affinity purified pHisBot preparation (after pH neutralization) using the LAL assay (Associates of Cape Cod) detected no significant endotoxin contamination. The assay was performed using the endpoint chromogenic method (without diazo-coupling) according to the manufacturer's instructions. This method can detect concentrations of endotoxin greater than or equal to 0.03 EU/ml (EU refers to endotoxin units). The LAL assay was run using 0.5 ml of a solution comprising 0.5 mg pHisBot protein in 50 mM NaHPO.sub.4, pH 7.0, 0.3 M NaCl, 10% glycerol; 30-60 EU were detected in the 0.5 ml sample. Therefore, the affinity purified pHisBot preparation contains 60-120 EU/mg of protein. FDA Guidelines for the administration of parenteral drugs require that a composition to be administered to a human contain less than 5 EU/kg body weight (The average human body weight is 70 kg; therefore up to 349 EU units can be delivered in a parental dose.). Because very small amount of protein are administered in a vaccine preparation (generally in the range of 10-500 .mu.g of protein), administration of affinity purified pHisBot containing 60-120 EU/mg protein would result in delivery of only a small percentage of the permissible endotoxin load. For example, administration of 10-500 .mu.g of purified pHisBot to a 70 kg human, where the protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU [i.e., 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose (less than 5 EU/kg body weight)].

Detailed Description Text (646):
The results shown in Example 24d demonstrated that the pHisBot protein is an excellent candidate for use as a vaccine as it could be produced as a soluble protein in E. coli and could be purified free of pyrogen activity. In order to optimize the expression and purification of the pHisBot protein, a variety of growth and purification conditions were tested.

Detailed Description Text (649):
The influence of the host strain utilized upon the production of soluble pHisBot protein was investigated. A large scale purification of pHisBot was performed [as described in Example 24d above] using the BL21(DE3) host (Novagen) rather than the

BL21(DE3)pLysS host. The deletion of the pLysS plasmid in the BL21(DE3) host yielded higher levels of expression due to de-repression of the plasmid's T7-lac promoter. However, the yield of affinity-purified soluble recombinant protein was very low (approximately 600 .mu.g/liter culture) when purified under conditions identical to those described in Example 24d above. This result was due to the fact that expression in the BL21(DE3) host yielded very high level expression of the pHisBot protein as insoluble inclusion bodies as shown by SDS-PAGE analysis of protein prepared from induced BL21(DE3) cultures (FIG. 29, lanes 1-7, described below). These results demonstrate that the pHisBot protein is not inherently toxic to E. coli cells and can be expressed to high levels using the appropriate promoter/host combination.

Detailed Description Text (661):
For optimization of purification conditions, large scale cultures (3.times.1 liter) were grown at 30.degree. C. and induced with 1 mM IPTG for 5 hours as described above. The cultures were pooled, distributed to centrifuge bottles, cooled and pelleted as described in Example 24d. The cell pellets were frozen at -70.degree. C. until used. Each cell pellet represented 1/3 of a liter starting culture and individual bottles were utilized for each optimization experiment described below. This standardized the input bacteria used for each experiment, such that the yields of affinity purified pHisBot protein could be compared between different optimization experiments.

Detailed Description Text (663):
A lysate of pHisBot culture was prepared in PBS (pH 8.0) and applied to a 3 ml Ni-NTA column equilibrated in PBS (pH 8.0) using a flow rate of 0.2 ml/min (3-4 column volumes/hr) using an Econo chromatography system (BioRad). The column was washed with PBS (pH 8.0) until the absorbance (OD.sub.280) of the elute was at baseline levels. The flow rate was then increased to 2 ml/min and the column was equilibrated in PBS (pH 7.0). A pH gradient (pH 7.0 to 4.0 in PBS) was applied in order to elute the bound pHisBot protein from the column. Fractions were collected and aliquots were resolved on SDS-PAGE gels. The PAGE gels were subjected to Western blotting and the pHisBot protein was detected using a chicken anti-C. botulinum Type A toxoid antibody as described in Example 22.

Detailed Description Text (668):
The column was eluted using an imidazole step gradient [in 50 mM NaHPO.sub.4, 0.5M NaCl (pH 7.0)]. Elution steps were 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 200 mM, 1.0M imidazole, followed by a wash using 0.1 mM EDTA (to strip the nickel from the column and remove any remaining protein). In each step, the wash was continued until the OD.sub.280 returned to baseline. Fractions were resolved on SDS-PAGE gels, Western blotted, and pHisBot protein detected using a chicken anti-C. botulinum Type A toxoid antibody as described in Example 22. Duplicate gels were stained with Coomassie blue to detect eluted protein in each fraction.

Detailed Description Text (673):
Batch purifications were performed (as described in Example 24d) using several buffers to determine if alternative buffers could be utilized for binding of the pHisBot protein to the Ni-NTA column. It was determined that quantitative binding of pHisBot protein to the Ni-NTA resin was achieved in either Tris-HCl (pH 7.9) or NaHPO.sub.4 (pH 8.0) buffers. Binding of the pHisBot protein in NaHPO.sub.4 buffer was not inhibited using 5 mM, 8 mM or 60 mM imidazole. Quantitative elution of bound pHisBot protein was obtained in buffers containing 50 mM NaHPO.sub.4, 0.3M NaCl (pH 3.5-4.0), with or without 10% glycerol. However, quantitation of soluble affinity purified pHisBot protein before and after a freeze thaw (following several weeks storage of the affinity purified elute at -20.degree. C.) revealed that 94% of the protein was recovered using the glycerol-containing buffer, but only 68% of the protein was recovered when the buffer lacking glycerol was employed. This demonstrates that glycerol enhanced the solubility of the pHisBot protein in this low pH buffer when the eluted protein was stored at freezing temperatures (e.g., -20.degree. C.). Neutralization of pH by addition of NaH.sub.2 PO.sub.4 buffer did not result in obvious protein precipitation.

Detailed Description Text (675):
The reported high affinity interaction of the Ni-NTA resin with His-tagged proteins (K.sub.d =1.times.10.sup.-13 at pH 8.0) suggested that it should be possible to manipulate the resin-protein complexes without significant release of the bound protein. Indeed, it was determined that after the recombinant protein was bound to the Ni-NTA resin, the resin-pHisBot protein complex was highly stable and remained bound following repeated rounds of centrifugation of the resin for 2 min at 1600.times.g. When this centrifugation step was performed in a 50 ml tube (Falcon), a tight resin pellet formed. This allowed the removal of spent soluble lysate by pouring off the

supernatant followed by resuspension of the pellet in wash buffer. Further washes can be performed by centrifugation. The ability to perform additional washes permits the development of protocols for batch absorption of large volumes of lysate with removal of the lysate being performed simply by centrifugation following binding of the recombinant protein to the resin.

Detailed Description Text (677):
A pilot purification was performed following this protocol and yielded 18 mg/liter affinity-purified pHisBot. The pHisBot protein was greater than 90% pure as estimated by Coomassie staining of an SDS-PAGE gel. This represents the highest observed yield of soluble affinity-purified pHisBot protein and this protocol eliminates the need for separate imidazole-containing binding and wash buffers. In addition to providing a simplified and efficient protocol for the affinity purification of recombinant pHisBot protein, the above results provide a variety of purification conditions under which pHisBot protein can be isolated.

Detailed Description Text (680):
In Example 23 it was demonstrated that neutralizing antibodies are generated in mouse serum after nasal immunization with the pMBot protein. However, the pMBot protein was found to copurify with significant amounts of endotoxin which could not be easily removed. The pHisBot protein, in contrast, could be isolated free of significant endotoxin contamination making pHisBot a superior candidate for vaccine production. To further assess the suitability of pHisBot as a vaccine, the immunogenicity of the pHisBot protein was determined and a comparison of the relative immunogenicity of pMBot and pHisBot proteins in mice was performed as follows.

Detailed Description Text (681):
Two groups of eight BALBc mice were immunized with either pMBot protein or pHisBot protein using Gerbu GMDP adjuvant (CC Biotech). pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaHPO.sub.4, 0.3M NaCl, 10% glycerol, pH 4.0) was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received an IP injection of 100 .mu.l antigen/adjuvant mix (50 .mu.g antigen plus 1 .mu.g adjuvant) on day 0. Mice were boosted as described above with the exception that the route of administration was IM on day 14 and 28. The mice were bled on day 77 and anti-C. botulinum Type A toxoid titers were determined using serum collected from individual mice in each group (as described in Example 23). The results are shown in Table 40.

Detailed Description Text (682):
The results shown above in Table 40 demonstrate that both the pMBot and pHisBot proteins are immunogenic in mice as 100% of the mice (8/8) in each group seroconverted from non-immune to immune status. The results also show that the average titer of anti-C. botulinum Type A toxoid IgG is 2-3 fold higher after immunization with the pHisBot protein relative to immunization with the pMBot protein. This suggests that the pHisBot protein may be a superior immunogen to the pMBot protein.

Detailed Description Text (685):
The results shown in Example 26 demonstrated that both the pHisBot and pMBot proteins were capable of inducing high titers of anti-C. botulinum type A toxoid-reactive antibodies in immunized hosts. The ability of the immune sera from mice immunized with either the pHisBot or pMBot proteins to neutralize C. botulinum type A toxoid in vivo was determined using the mouse neutralization assay described in Example 23b.

Detailed Description Text (688):
These results demonstrate that antibodies capable of neutralizing C. botulinum type A toxin are induced when either of the recombinant C. botulinum C fragment proteins pHisBot or pMBot are used as immunogens.

Detailed Description Text (695):
This example involved: (a) construction of pUC1960-2680 and characterization of the expressed protein by Western blot analysis; (b) cloning and expression of the 1960-2680 interval as an affinity tagged protein in pET and pMal vectors and (c) affinity purification and characterization of soluble MBP tagged proteins from clones expressing the 1870-2680, 1870-2190 or 1960-2680 intervals.

Detailed Description Text (700):
The protein samples were resolved by electrophoresis on a 12.5% SDS-PAGE gel and the proteins detected either by Coomassie blue staining (detects all proteins) and Western blot analysis (detects specific proteins) utilizing a goat anti-toxin A specific antibody (TechLabs) as follows. The 12.5% SDS-PAGE gels were loaded with the protein

samples. After electrophoresis, the gel was bisected. One half was stained with Coomassie blue and the proteins on the other half were transferred to a solid support for Western blot analysis. Protein transfer was confirmed by Ponceau S staining (as described in Example 12b). The blot was then incubated for 1 hr at 20.degree. C. in PBS containing 0.1% Tween 20 (PBST) and 5% milk (blocking buffer). Then 10 ml of a solution comprising a 1/1000 dilution of an affinity purified goat anti-C. difficile toxin A antibody (Tech Labs) in blocking buffer was added and the blot was incubated for 1 hr at room temperature. The blot was then washed and the presence of the bound anti-C. difficile antibody was detected using a rabbit anti-goat alkaline phosphatase conjugate as secondary antibody as described in Example 3. The resulting Coomassie blue-stained gel and developed Western blot are shown in FIG. 32.

Detailed Description Text (702):
b) Cloning and Expression of the 1960-2680 Interval as an Affinity Tagged Protein in pET and pMal Vectors

Detailed Description Text (703):
As shown above, the protein produced by the pUC1960-2680 construct was unstable (i.e., prone to proteolytic degradation) and furthermore, it lacks an affinity tag. The instability of the pUC1960-2680 protein may be due to the presence of the .alpha.-peptide of the lacZ gene at the C terminus of the fusion protein; the presence of these sequences on a fusion protein is known to results in the production of an unstable protein. In order to determine whether soluble, stable, affinity purified fusion protein representing the pUC1960-2680 interval could be isolated, the following two constructs were made. The pPA1960-2680 construct contains the 1960-2680 interval of C. difficile toxin A in the pET23c vector (Novagen). The pET23 series of vectors permits the expression of inserted genes as a fusion protein containing a poly-histidine tag or tract at either the C- or N-terminus of the fusion protein; the pPA1960-2680 construct expresses the C. difficile toxin A repeat region as a fusion protein containing a C-terminal poly-histidine tract. The pMA1960-2680 construct contains the 1960-2680 interval of C. difficile toxin A in the pMal-c vector (New England BioLabs) and expresses a fusion protein comprising the MBP at the N-terminus of the fusion protein.

Detailed Description Text (709):
c) Affinity Purification and Characterization of Soluble MBP-Tagged Proteins From Constructs Expressing the 1870-2680, 1870-2190 or 1960-2680 Intervals of C. difficile Toxin A

Detailed Description Text (710):
Large scale (1 liter) cultures of the pMal-c vector (i.e., vector lacking an insert), and each of the following recombinant constructs were grown, induced, and soluble protein fractions isolated: pMA1870-2190 (Example 17), pMA1960-2680 (Example 28b) and pMA1870-2680 [Example 11; Interval 6; Interval 6 contains amino acid residues 1873 through 2684 (SEQ ID NO:29) of the C. difficile toxin A protein]. The large scale cultures were grown at 32.degree. C. in 2.times. YT broth and recombinant protein expression was induced by the addition of IPTG to 0.3 mM at OD.sub.600 of 0.6. The cultures were induced for 4-5 hrs and then the cells were harvested. Soluble protein extracts were prepared and subjected to affinity chromatography to isolate recombinant fusion protein (Example 11d), and analyzed by Coomassie staining and Western analysis as described (Example 11b).

Detailed Description Text (711):
Briefly, soluble extracts were prepared and applied in PBS to an amylose resin (New England Biolabs) column. The column was eluted with PBS containing 10 mM maltose. Protein yields were 40 mg per 1 liter starting volume (i.e., 1 liter cultures) for each recombinant. Protein samples were analyzed by electrophoresis on 7.5% SDS-PAGE gels followed by staining with Coomassie blue and Western blot analysis as described in section a). Protein samples were prepared for electrophoresis by mixing 1 .mu.l total (T) or soluble (S) protein with 4 .mu.l PBS and 5 .mu.l 2.times. sample buffer, or 5 .mu.l eluted (E) protein and 5 .mu.l 2.times. sample buffer or 0.5 .mu.l eluted protein, 4.5 .mu.l PBS and 5 .mu.l 2.times. sample buffer (1/10E). Samples of pMA1870-2680 and pPA1870-2680 (inclusion body preparations described in Example 11) were also resolved on the gel. The samples were heated to 95.degree. C. for 5 min, then cooled and loaded on a 7.5% SDS-PAGE gel. Broad range molecular weight protein markers (BioRad) were also loaded to allow estimation of the MW of identified fusion proteins.

Detailed Description Text (715):
1) That the pMA1870-2190 protein was unstable but was at least partially soluble under

the growth conditions utilized. The affinity purified pMA1870-2190 preparation does however contain significant concentrations of full length fusion protein (FIG. 33, lane 2).

Detailed Description Text (716):
2) The pMA1960-2680 protein was partially soluble (compare lanes 3' and 4' in FIG. 33) and the integrity of the affinity purified protein (FIG. 33, lanes 5' and 6') was comparable to that of the pMA1870-2680 preparation (FIG. 33, lane 2).

Detailed Description Text (718):
The results shown above provide a method for the production of affinity purified recombinant C. difficile toxin A protein from the 1870-2190 and 1960-2680 intervals. These results are in contrast to those obtained when using the pUC1960-2680 construct, which was prepared according to the description of Lyerly et al. [(1990) Curr. Microbiol. 21:29]. The protein expressed by the pUC1960-2680 construct was mainly insoluble and could not be affinity purified due to the absence of an affinity tag on the recombinant protein.

Detailed Description Text (721):
For potential utilization as a human vaccine (i.e., to induce active immunity) or as an antigen in a host animal to induce protective antibodies (i.e., antitoxin) for passive immunization of humans, a protein antigen should be 1) easily purified, 2) well characterized and of a high purity, 3) pyrogen poor (when used as a human vaccine), 4) immunogenic and 5) capable of inducing a protective immune response. In the case of the C. difficile toxin A repeat antigen, the protein must be soluble and capable of assuming a conformation which will induce a protective response. As was shown in Example 17, when pPA1870-2680(N/C) protein, which was expressed as insoluble protein inside inclusion bodies, was solubilized with SDS and then used to immunize chickens, no protective anti-toxin A antibodies were produced.

Detailed Description Text (722):
In this example, the recombinant C. difficile toxin A proteins were expressed and evaluated as vaccine candidates using the criteria stated above. This example involved a) evaluation of the utility of affinity purified pMA1870-2680 protein as a vaccine antigen, b)construction, purification and evaluation of the pGA1870-2680 protein, c) development of a protocol for production of soluble pPA1870-2680, d) construction of pPA1870-2680(N) and large scale purification of N, C and N/C his-tagged 1870-2680 protein, e) construction of pPTrxA1870-2680(N) (C)and (N/C), and large scale purification of N, C and N/C his-tagged Trx 1870-2680 proteins, f) large scale affinity purification of pPA1870-2680 and pPB1750-2360 proteins and determination of endotoxin levels and g) construction, large scale affinity purification of pPB1750-2360(N/C) and determination of endotoxin levels.

Detailed Description Text (723):
a) Evaluation of the Utility of Affinity Purified pMA1870-2680 Protein as a Vaccine Antigen

Detailed Description Text (724):
Although the pMA1870-2680 protein (Example 11) was shown to be easily purified, immunogenic and capable of inducing a protective response (Example 17), the ability to use this protein as a vaccine is limited by the poor purity of the affinity purified protein (see FIG. 33, lanes 7' and 8'). It was estimated that only 50% of the affinity purified protein represents full-length fusion protein. The remainder of the proteins in the affinity purified preparation was found to be primarily MBP alone and contaminating E. coli proteins.

Detailed Description Text (725):
In order to assess whether affinity purified pMA1870-2680 protein could be used as a vaccine candidate, the endotoxin content in two independently affinity purified preparations of pMA1870-2680 protein was determined. Pyrogen content in the samples was assayed utilizing the Limulus assay (LAL kit; Associates of Cape Cod) as described in Example 24d. Both samples of affinity purified pMA1870-2680 were found to contain high levels of endotoxin (>50,000 EU/mg purified recombinant protein). As seen in Examples 24a and b, high endotoxin load was determined to be a general feature of affinity purified MBP fusion proteins, or MBP alone. The above results indicate that, using current purification protocols, affinity purified MBP-C. difficile toxin A fusion proteins are not suitable for use as vaccine antigens.

Detailed Description Text (726):

The pMA1870-2680 expression construct was designed to facilitate purification of the toxin A protein from the MBP tag by cleavage of the fusion protein at the engineered Factor Xa cleavage site located between the MBP and toxin A protein domains. The feasibility of obtaining substantially endotoxin-free, soluble recombinant C. difficile toxin A protein by purification of cleaved C. difficile toxin A protein from the MBP-toxin A fusion protein was assessed. Factor Xa (New England Biolabs) was added to the affinity purified pMA1870-2680 protein (0, 0.2, 0.5, 1.0 and 2.5% Factor Xa/pMA1870-2680 protein ratio) in PBS containing 10 mM maltose and the mixtures were incubated for 5.5 and 20 hrs at room temperature. The extent of cleavage was assessed by Coomassie blue staining proteins after electrophoresis on SDS-PAGE gels. The results demonstrated that some cleavage was observed in the 2.5% Factor Xa sample after 20 hrs, but cleavage was only partial. This indicates that cleavage of pMA1870-2680 is not an efficient purification strategy to obtain soluble endotoxin-free C. difficile toxin A repeat protein using the above tested reaction conditions.

Detailed Description Text (728):
In order to facilitate evaluation of the GST-containing proteins as a means of large scale production of antigens, the C. difficile toxin A repeats were expressed as a fusion with GST. The C. difficile toxin A repeats were isolated by cleavage of pPA1100-2680 (Example 11) with SpeI followed by treatment with the Klenow fragment to fill in the ends; the DNA was then digested with XhoI. The SpeI (Klenow filled)-XhoI fragment was cloned into EcoRI (Klenow filled)-XhoI cleaved pGEX3T vector (Pharmacia) to yield the pGA1870-2680 expression construct.

Detailed Description Text (729):
A large scale (1 liter) 2.times. YT culture of pGA1870-2680 [in BL21 host cells (Novagen)] was grown in 2.times. YT medium containing 50 .mu..mu.g/ml ampicillin and induced (using IPTG to 1.0 mM) for 3 hrs at 30.degree. C. as described in Example 28. A soluble lysate of the pGA1870-2680 large scale culture (resuspended in PBS) was prepared, and used to affinity purify soluble affinity tagged protein. The pGA1870-2680 lysate was affinity purified on Glutathione-agarose resin (Pharmacia) as described in [Smith and Corcoran, Current Protocols in Molecular Biology, Suppl. 28 (1994) pp. 16.7.1-16.7.7] with the exception that binding of protein to resin was for 1 hr at 4.degree. C. Briefly, following induction of the 1 liter culture for 3 hrs, the cells were collected by centrifugation for 10 min at 5,000 .times. g at room temperature. The cell pellet was resuspended in 10 ml ice-cold PBS. The cells were then disrupted by sonication as described in Example 24d. Triton X-100 was added to a final concentration of 1% and the sample was well mixed. Insoluble debris was removed by centrifugation of the sample for 5 min at 10,000 .times. g at 4.degree. C. The supernatant was carefully removed and added to 1 ml of 50% slurry of glutathione-agarose beads (Pharmacia). The mixture was allowed incubate for 1 hr at 4.degree. C. to allow the GST-tagged fusion protein to bind to the resin. The glutathione-agarose beads were then washed by adding 50 ml of ice-cold PBS, mixing and centrifuging for 10 sec at 500 .times. g at room temperature. The wash step was repeated twice (for a total of 3 washes). The resin was resuspended in 1 ml of ice-cold PBS and transferred to a 1.5 ml microcentrifuge tube. The resin was pelleted by centrifugation for 10 sec at 500 .times. g at room temperature. The supernatant was removed and the fusion protein was eluted from the washed resin by adding 1 ml of 50 mM Tris-HCl (pH 8.0) and 5 mM reduced glutathione. The tube was mixed gently for 2 min then centrifuged for 10 sec at 500 .times. g at room temperature. The elution was repeated twice and the supernatants were pooled. The pooled supernatant, containing the eluted fusion protein, was stored in a solution containing 50 mM Tris-HCl (pH 8.0), 5 mM reduced glutathione and 10% glycerol. Endotoxin content of the purified fusion protein was determined using the LAL kit as described in Example 24d.

Detailed Description Text (730):
Samples from the growth, induction and purification steps (uninduced, induced, total, soluble, and affinity purified elution) were resolved on SDS-PAGE gels, and proteins detected by staining with Coomassie blue (as described in Example 28). The fusion protein was found to be partially soluble (i.e., most protein remained in the pellet) and approximately 0.5 mg/liter starting culture of mostly full length protein was affinity purified. The affinity purified preparation contained approximately 5000 EU/mg of affinity purified fusion protein. These results demonstrate that under the above conditions, the pGEX expression system did not facilitate high level production of recombinant C. difficile toxin A fusion protein, and that the recovered protein contained significant endotoxin contamination.

Detailed Description Text (732):
In Example 11 it was shown that, when produced by growth at 37.degree. C., induced

pPA1870-2680 protein is almost entirely insoluble. To determine if expression at a lower temperature could enhance solubility, a culture of pPA1870-2680(N/C) was grown at 30.degree. C. and the level of soluble affinity purifiable protein determined. A soluble lysate (in Novagen 1.times. binding buffer) from an induced 1 liter 2.times. YT culture was prepared as described below.

Detailed Description Text (734):
Samples of total, soluble, and eluted protein were resolved by SDS-PAGE. Total protein was detected by staining the gel with Coomassie blue. The purification resulted in a yield of 34 mg of affinity purified protein from a 1 liter starting culture (3.2% of the total soluble extract), of which at least 90-95% of the protein was found to migrated as a single band of the predicted MW (90 kd) for the recombinant C. difficile toxin A fusion protein [i.e., the pPA1870-2680(N/C) protein].

Detailed Description Text (737):
Expression plasmids that facilitated expression of the 1870-2680 interval of C. difficile toxin A with either a N-terminal his-tag [pPA1870-2680 (N)], a C terminal his-tag [pPA1870-2680(C)] or with both N- and C-terminal his-tags [pPA1870-2680(N/C)] were evaluated for large scale production and affinity purification of C. difficile toxin A repeat protein.

Detailed Description Text (742):
Large scale cultures of pPA1870-2680(N) and pPA1870-2680(C) were grown (using the BL21(DE3)pLysS host), induced and soluble protein was affinity purified and eluted as described in section c) above. In each case 10-20 mg affinity purified protein was recovered and the purified protein was found to be greater than 50% full length fusion protein as estimated by SDS-PAGE analysis. However, the bulk of the pPA1860-2680(C) protein eluted in the 40 mM wash buffer. In an attempt to identify wash conditions which did not result in the elution of significant amounts of the pPA1860-2680(C) protein, the following experiment was performed.

Detailed Description Text (744):
The above results demonstrated that soluble, affinity purified C. difficile toxin A protein was isolated using any of the pPA1870-2680 (N), (C), or (N/C) expression plasmids. p1 e) Construction of pPTrxA1870-2680(N) (C) and (N/C) and Large Scale Purification of N, C and N/C His-Tagged Trx 1870-2680 Proteins

Detailed Description Text (745):
The thioredoxin (Trx) expression system (Invitrogen) has been developed to facilitate soluble expression of normally insoluble or difficult to express proteins. Genes are cloned into the pTrxFus vector and expressed as fusions with the E. coli thioredoxin protein; this linkage often confers the solubility properties of thioredoxin to the fusion protein [La Vallie, et al. (1993) Bio/Technology 11:187]. However, the pTrxFus vector has several undesirable properties for an expression vector. All plasmids must be grown in specific strains and growth media since fusion protein expression in this system is inducible by tryptophan. As well, the promoter is not stringently controlled, such that low level expression of fusion protein occurs at reduced temperatures (i.e., 30.degree. C.). Finally, the expression vector does not contain an affinity tag to facilitate high level affinity purification of soluble fusion protein.

Detailed Description Text (746):
To facilitate construction of IPTG-inducible, affinity tagged Trx fusion proteins, the pETHisTrx vector was constructed. The thioredoxin gene of pTrxFus (Invitrogen) was excised as an NdeI-BamHI DNA fragment and was cloned into NdeI-BamHI digested pETHisb vector (Example 18) to created the pETHisTrx vector.

Detailed Description Text (747):
In the pETHisTrx vector, the Trx gene is expressed from the pET16b promoter and contains the pET16b N-terminal leader and his-tag sequence upstream of Trx, and the pET23b polylinker (from the BamHI site) downstream of the Trx gene for construction of C-terminal genetic fusions. Three expression constructs which facilitate expression of a Trx-toxin A 1870-2680 interval fusion, as N, C or N/C terminal his-tags were constructed as follows.

Detailed Description Text (748):
The pPTrxA1870-2680(N/C) construct was constructed by ligation of the NdeI-BamHI (filled) Trx gene (isolated from the pTrxFus vector) and a SpeI (filled)-XhoI fragment containing the C. difficile toxin A 1870-2680 gene [isolated from pPA1100-2680 construct (Example 11)] into the NdeI-XhoI cleaved pETHisb vector (the filled BamHI and

SpeI sites blunt end ligate together and create an in-frame Trx-C. difficile toxin A fusion).

Detailed Description Text (749):
The above Trx-C. difficile toxin A fusion was excised as an NdeI-HindIII fragment and inserted into NdeI-HindIII cleaved pET23a vector (Novagen) to create pPTrxA1870-2680(C).

Detailed Description Text (752):
Large scale cultures of all three TrxA1870-2680 fusions [i.e., pPTrxA1870-2680(C), pPTrxA1870-2680(N) and pPTrxA1870-2680(N/C)] were grown and soluble affinity purified protein was isolated as described in section c) above. In all cases, affinity purified Trx fusion protein yields were similar in terms of solubility, mg/liter culture yields, and purity to the parallel pPA1870-2680 N, C, or N/C constructs described in section d) above.

Detailed Description Text (753):
f) Large Scale Affinity Purification of pPA1870-2680 and pPB1750-2360 Proteins and Determination of Endotoxin Levels

Detailed Description Text (754):
Preparations of affinity purified pPA1870-2680(N/C) (Example 15) and pPB1750-2360 (Example 15b) protein were generated to determine the endotoxin levels in the purified samples. All buffers were filter sterilized and gloves were worn through the preparation of the buffers to reduce buffer-mediated endotoxin contamination of the purified recombinant protein samples. Large scale purifications of pPA1870-2680(N/C) and pPB1750-2360 proteins were performed as follows.

Detailed Description Text (756):
FIG. 34 shows a Coomassie blue-stained gel showing the steps of the purification. Protein samples were prepared for electrophoresis by mixing 1 .mu.l total (T) or soluble (S) or soluble protein after binding to NiNTA resin and centrifugation (A) protein with 4 .mu.l PBS and 5 .mu.l 2.times. SDS-PAGE sample buffer, or 5 .mu.l eluted (E) protein and 5 .mu.l 2.times. sample buffer. The samples were heated to 95.degree. C. for 5 min, then cooled and loaded onto a 7.5% SDS-PAGE gel. Broad range molecular weight protein markers (M; BioRad) were also loaded, to allow the estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected generally by staining the gel with Coomassie blue. In FIG. 34, lanes 1-4 contain protein from the purification of the pPA1870-2680 protein and lanes 5-8 contain protein from the purification of the pPB1750-2360 protein.

Detailed Description Text (757):
The purification resulted in a yield of approximately 30 mg/liter of affinity purified protein from 1 liter starting cultures (2-2.5% of the total soluble extract) for both proteins, of which at least 90-95% of the protein migrated as a single band of the predicted MW (90 kD) for the recombinant C. difficile toxin A protein. In both cases, most (i.e, greater than 90%) of the induced protein was soluble, and bound the resin quantitatively under the purification conditions utilized.

Detailed Description Text (759):
g) Construction, Large Scale Affinity Purification of pPB1750-2360(N/C) and Determination of Endotoxin Levels

Detailed Description Text (760):
As shown above, the affinity purified pPB1750-2360 protein contained higher levels of endotoxin than did the purified pPA1870-2680(N/C) protein. The pPB1750-2360 protein contains a poly-histidine tract at the carboxy-terminus while pPA1870-2680(N/C) contains a poly-histidine tract at both the amino- and carboxy-termini. The presence of a poly-histidine tract at both ends of the fusion protein permitted higher stringency wash conditions to be employed during the affinity purification of pPA1870-2680(N/C) as compared to pPB1750-2360 (40 mM imidazole versus 20 mM imidazole, respectively).

Detailed Description Text (761):
In order to produce a fusion protein comprising the 1750-2360 interval of C. difficile toxin B containing poly-histidine tracts at both the amino- and carboxy-termini, pPB 1750-2360(N/C) was constructed as follows. pPB 1750-2360 (Example 15b) was digested with NdeI and XhoI and the 1.5 kb NdeI/XhoI fragment was isolated and inserted into pETHisb vector (Example 18) digested with NdeI and XhoI. Routine procedures were employed for this construction as described in the preceding Examples.

Detailed Description Text (764):
Analysis of the eluted pPB1750-2360(N/C) on SDS-PAGE gels stained with Coomassie blue revealed a single band of the MW expected for the full-length fusion protein.

Detailed Description Text (765):
The endotoxin levels of the purified pPB1750-2360(N/C) protein was determined using the LAL kit (Example 24d). Three separate determinations were conducted and the endotoxin level was found to be 80, 300 or 450 EU/mg of purified recombinant protein. While not limited to any particular mechanism, it is believed that the inconsistent LAL assay results seen with pPB1750-2360(N/C) and the high reading obtained with pPB1750-2360 (see section f) are due to non-specific agglutination of the LAL components by carbohydrate binding moieties present on the C. difficile toxin B sequences present on these proteins. Regardless of whether the actual endotoxin level is 80 or 450 EU/mg purified protein, the affinity purified pPB1750-2360(N/C) preparation represents a substantially endotoxin-free preparation of recombinant protein (Administration of 10 to 500 .mu.g of purified pPB1750-2360(N/C) would result in the introduction of only 4.5 to 225 EU; in a 70 kg human this amount of endotoxin is 1.3 to 64.5% of the maximum permissible dose).

Detailed Description Text (766):
The above results provide a protocol for the affinity purification of substantially endotoxin-free preparations of recombinant C. difficile toxin A and B repeat proteins in high yields.

Detailed Description Text (771):
Previous attempts to produce soluble affinity purified protein utilizing the pPA1870-2190 (Example 17a) or pPA1960-2680 (Example 28) vectors were unsuccessful, as assessed by analysis of total and soluble protein produced in small scale cultures. However, the solubility properties of a protein determined utilizing small scale or minicultures may not translate to large scale cultures, due to differences in buffers, sonication conditions, etc. Indeed, the successful expression of soluble, substantially endotoxin-free C. difficile toxin A repeat protein utilizing the pPA1870-2680 N, C or N/C constructs suggested that the conditions utilized to solubilize these proteins might also enhance solubility of the pPA1870-2190 and pPA1960-2680 proteins. This hypothesis was tested as follows.

Detailed Description Text (772):
Large scale cultures of pPA1870-2190 and pPA1960-2680 were grown and soluble protein affinity purified on Ni-NTA resin as described in Example 29c. Both the BL21(DE3) and BL21(DE3)pLysS hosts for pPA1960-2680, and the BL21(DE3)pLysS host for pPA1870-2190 were utilized. The culture of pPA1870-2680(N/C) [in the BL21(DE3)pLysS host] was grown at 30.degree. C. to an OD.sub.600 of 0.9 in 1 liter of 2.times. YT medium containing 100 .mu.g/ml ampicillin and 0.2% glucose; when the host utilized harbored the pLysS plasmid, 34 .mu.g/ml chloramphenicol was added to the above medium. Protein expression was induced by addition of IPTG to 1 mM. After 5 hrs of induction, the cells were cooled for 15 min in a ice water bath and then centrifuged for 10 min at 5,000 rpm in a JA10 rotor (Beckman) at 4.degree. C. The pellets were resuspended in a total volume of 40 mls Novagen 1.times. binding buffer (5 mM imidazole, 0.5M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70.degree. C. for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4.times.20 second bursts using a Branson Sonifer 450 with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 .times. g) in a JA-17 rotor (Beckman) at 4.degree. C. The soluble lysate (after addition of NP40 to 0.1%) was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin (Qiagen): Novagen 1.times. binding buffer by stirring for 3 hr at 4.degree. C. The slurry was poured into a 1 cm internal diameter column (BioRad), and washed with the following solutions in succession: 15 mls Novagen 1.times. binding buffer containing 0.1% NP40, 15 ml Novagen 1.times. binding buffer, 15 ml wash buffer (40 mM imidazole, 0.5M NaCl, 20 mM Tris-HCl, pH 7.9). The bound protein was eluted in 200 mM imidazole, 0.5M NaCl, 20 mM Tris-HCl, pH 7.9.

Detailed Description Text (773):
Samples of total, soluble, and eluted protein (both the 40 mM and 200 mM wash and elution buffers) were resolved by SDS-PAGE. Total protein was detected by Coomassie staining, and C. difficile toxin A-reactive protein (in the case of pPA1960-2680) detected by Western blot detection, utilizing affinity purified goat anti-toxin A antibody as described in Example 28.

Detailed Description Text (774):
The results of these analyses showed that for the pPA1870-2190 protein, only 600 .mu.g protein/liter culture was purified in the 200 mM imidazole elution. The C. difficile toxin A protein was expressed to high levels with this construct, but most of the induced protein was insoluble. As well, the pPA1870-2190 protein represented less than 10% of the total eluted protein. For the pPA1960-2680 construct, total yields of soluble affinity purified protein was either 1 mg [B121(DE3)pLysS host] or 200 .mu.g [BL21(DE3) host] in the 200 mM elution fraction. Coomassie and Western analysis demonstrated that the pPA1960-2680 protein was expressed to high levels, but that most of the induced protein was insoluble, and that eluted protein preparations contained only approximately 20% C. difficile toxin A-reactive protein.

Detailed Description Text (777):
To determine if the solubility of recombinant proteins comprising 1870-2680 interval of C. difficile toxin A could be enhanced by utilizing the solubilizing properties of the Trx protein, a fusion construct in which the 1870-2680 interval was expressed as a fusion to thioredoxin (Trx) was constructed.

Detailed Description Text (778):
The pPTrxA1870-2190 construct was made in two steps. First, the 1870-2190 interval was cloned into the pTrxFus vector (Invitrogen). This was accomplished by ligating the KpnI-SalI fragment from pMA1870-2190 which contains the 1870-2190 interval of C. difficile toxin A into the KpnI-SalI cleaved pTrxFus vector. A recombinant clone containing the appropriate DNA fragments was selected and the sequences encoding the Trx-C. difficile toxin A fusion protein were excised utilizing NdeI and SalI, and cloned into the pETHisb vector (Example 18) cleaved with NdeI and XhoI. The resultant construct, pPTrxA1870-2190, comains an N-terminal his-tagged Trx-C. difficile toxin A fusion driven by the pET16b promoter.

Detailed Description Text (779):
Purification of soluble affinity purified Trx-C. difficile toxin A protein from the pPTrxA1870-2190 construct was performed from a large scale culture as described in section a) above. Total, soluble and elution samples were resolved on a 12.5% SDS-PAGE gel and protein was detected by staining with Coomassie blue.

Detailed Description Text (780):
The results of this analysis revealed that the total yield of affinity purified recombinant protein was 2 mg of greater than 50% pure protein in the 200 mM imidazole elution. This yield of 1 mg specific protein (50% of 2 mg total purified protein) represents a ten fold increase over the yield obtained with the pPA1870-2190 construct (10% of 600 .mu.g, or less than 100 .mu.g specific protein) and demonstrates the solubilizing property of the Trx protein. However, the majority of induced protein was insoluble with both constructs (i.e., pPTrxA1870-2190 and pPA1870-2190) and the overall affinity purifiable protein yield with the pPTrxA1870-2190 vector was still less than 20 fold lower that obtained with the pPA 1870-2680 constructs.

Detailed Description Text (788):
Microtiter plates were coated at 100 .mu.l/well with either 0.05 .mu.g/ml of the recombinant C. difficile toxin A protein, pPA1870-2680 (Example 11) or 1 .mu.g/ml of the recombinant C. difficile toxin B, pPB1750-2360 (Example 18b). The ELISA was performed as described in Example 13c. The results of this analysis revealed that the antibody titers were both greater than 1:125,000. (Antibody titer is defined as the reciprocal of the highest antibody dilution that gives an ELISA signal that is at least 3-fold over pre-immune IgY.) The amount of specific anti-recombinant toxin A and anti-recombinant toxin B IgY was determined by affinity purification as described in Example 15c. The amount of specific anti-recombinant C. difficile toxin A and B antibodies present in the anti-pMA1870-2680 and anti-pPB1750-2360 preparations was determined to be about 160 .mu.g/ml and 200 .mu.g/ml, respectively.

Detailed Description Text (810):
Three hours after inoculation (Day 1), treatment was initiated for both groups. The groups were each orally treated using an 18 gauge feeding needle to administer 2 mls of a 4.times. concentrate of either pre-immune IgY or specific immune IgY against either the recombinant C. difficile toxin A (pMA1870-2680; Interval A-6) or toxin B (pPB1750-2360; Interval B-3). On Day 1, the hamsters were treated additionally two more times at 2 hour intervals. On Day 2, through 4 the hamsters were each treated with 2 mls of the respective antibody preparations 3 times daily roughly at 4 hour intervals. Each 2 ml dose contained about 40 mg of IgY of which about 400 .mu.g is specific IgY (determined by affinity purification as described in Example 15c) to the recombinant

toxin protein or about 1200 .mu.g of specific anti-C. difficile toxin protein per day. All animals were observed for the onset of diarrhea and death during and after the treatment period. The results are shown in FIG. 36.

Detailed Description Text (815):
A second experiment was conducted basically as described above with the exception that a mixture of antibodies to both recombinant C. difficile toxins A and B was tested for the ability to protect hamsters from CDAD. Equal volumes of an 8.times. concentration of IgYs to both recombinants (pMA1870-2680 and pPB1750-2360) were mixed to give a final concentration to each recombinant equal to 4.times.. Each dose (2 ml) contained approximately 80 mg/ml protein containing about 400 .mu.g of specific IgY (1% specific anti-C. difficile toxin protein as compared to the total) to each recombinant. The amount of specific anti-recombinant IgY to each toxin recombinant was determined by affinity purification using the respective recombinant protein. The resulting preparation therefore contains the same final concentration of anti-recombinant toxin A used in the previous experiment (section c(i) above) except it contains twice the amount of protein. Because of this difference, an additional test group was set-up and treated with equal volumes of two 8.times. concentration of anti-recombinant C. difficile toxin A and pre-immune IgY. As a control, a third group of hamsters were treated with an 8.times. concentrate of only pre-immune IgY. Nine hamsters per group were infected with 1.times.10.sup.4 C. difficile organisms (ATCC 43596) and then were treated 4 hours later with 2 mls of either preimmune IgY, anti-recombinant C. difficile toxin A IgY mixed with preimmune IgY or a mixture of anti-recombinant C. difficile toxin A and B IgYs. The animals were treated as described (section c(i) above) at 3 times a day for 4 days. The outcome of this experiment is shown in FIG. 37.

Detailed Description Text (836):
Three groups of egg-laying Leghorn hens were immunized with different toxin A recombinants proteins produced in the pMal vector. All were expressed as MBP fusions. They were pMA1870-2190 (Example 17), pMA1960-2680 (Example 28) and pMA1870-2680 (Example 11). The first two recombinants proteins comprise overlapping sub-fragments within the interval contained in the recombinant pMA1870-2680.

Detailed Description Text (852):
Anti-recombinant toxin A antibody levels generated using the different adjuvants were compared by ELISA. About 1 week after the last boost, at least 3 eggs from each of the 8 groups along with pre-immune eggs were collected, yolks pooled within the group and IgYs were extracted by PEG as described in Example 1. The purified anti-recombinant toxin A IgYs were then resuspended in PBS at 1.times. yolk volume. The protein concentration of each of the preparations, determined by absorbance at 280 nm, were all similar at about 4 to 5 mg/ml. The IgY reactivity and titer of each of the individual antibody preparations against pMA1870-2680 were determined by ELISA against a soluble (pPTrxA1870-2680N/C; Example 29) and an insoluble (pPA1870-2190; Example 17a) analog of the C. difficile toxin A 1870-2680 interval. These recombinant C. difficile toxin A analogs were used to coat the microtiter plates for ELISA instead of the recombinant used in the immunization (pMA 1870-2680) as both pPTrxA1870-2680N/C and pPA 1870-2680 were not expressed as fusions with the MBP as was the pMA1870-2680 immunogen. This was done in order to determine antibody reactivity against the toxin portion of the C. difficile toxin A recombinant specifically rather than to the MBP portion of the fusion protein.

Detailed Description Text (853):
The soluble analog pPTrxA1870-2680N/C used to coat the microtiter plate was expressed as a fusion with thioredoxin which aids in solubility and the resulting fusion protein probably exists in a native conformation. The insoluble analog pPA1870-2190, which presumably contains denatured epitopes, was also used to coat microtiter plates. The ELISA reactivity of each IgY to both the soluble and insoluble analogs was tested to determine if there was any preferential reactivity to one or the other analogs when different adjuvants were used for the generation of the IgY.

Detailed Description Text (866):
Egg-laying Leghorn hens were each immunized with the C. difficile toxin A recombinant pPA1870-2680(N/C) (Example 29d). This recombinant protein is expressed in the pET vector and was shown to be capable of isolation in a highly pure form which contained very low levels of endotoxin as compared to the same region expressed in other vectors such as pMal-c (Example 11). These results showed that the pPA1870-2680 recombinant protein would be compatible for use in a vaccine. Accordingly, the ability of pPA1870-2680 to stimulate an antibody response was tested.

Detailed Description Text (874):
As shown in Table 44, both the Freund's and RIBI adjuvants used in conjunction with pPA1870-2680(N/C) were able to elicit in vivo neutralizing antibodies against C. difficile toxin A as compared to pre-immune IgY. The ability of the antibodies to neutralize C. difficile toxin A shown in this example and in Example 35 appears to correlate well with their ELISA reactivity to a soluble (native) recombinant protein. These results show that the C. difficile toxin A recombinant, pPA1870-2680(N/C), was immunogenic in hens and was capable of generating in vivo neutralizing antibodies; therefore, the pPA1870-2680(N/C) protein is an excellent vaccine candidate.

Detailed Description Text (901):
Three groups of 90-100 gram female Golden Syrian hamsters (Charles River), each group containing 9 to 11 members, were tested as follows. Hamsters from each group were individually tagged using an ear punch for identification. The animals from each group were housed together and were given food and water ad libitum throughout the course of the experiment. Hamsters were immunized with two different recombinant C. difficile toxin A protein repeats fragments produced the in pMal-c vector and expressed with a maltose binding protein (MBP) fusion and one recombinant C. difficile toxin A protein repeats fragment produced the in pET vector. The animals were immunized subcutaneously with 25 .mu.g of purified protein of either pPA1870-2680N/C (Example 15), pMA1870-2680, a subfragment of pMA1870-2680 called pMA1960-2680 or the MBP (pMal-c) alone as a control. All three recombinant pMal vectors were grown and protein was expressed and purified as described in Example 28c. Recombinant pPA1870-2680N/C was purified as described in Example 29f.

Detailed Description Text (911):
The results shown in Table 46 demonstrate that protection against death occurred in some of the hamsters immunized with each of the recombinant toxin A proteins (i.e., pMA1960-2680 and pMA1870-2680). These results were not statistically significant compared to the fusion control (pMal-c which expresses only the MBP) at a P-value of 0.05 or less using Chi-squared analysis. Ninety percent mortality occurred in the fusion control immunized group (pMal-c). The percent mortality in the pMA1960-2680 immunized group was 38%. The percent mortality in the pMA1870-2680 immunized group was 70% and in the pPA1870-2680 immunized group was 81%. The time to death in recombinant C. difficile toxin A vaccinated group was not delayed compared to the control, occurring up to 3 days after infection. Necropsy of the dead hamsters revealed typical pathology such as severe megacecum.

Detailed Description Text (913):
The above results correlate with previously published work [Lyerly et al. (1990) Curr. Microbiol. 21:29] which showed that hamsters vaccinated with the smaller C. difficile toxin A recombinant fragment (the 1960-2680 interval) expressed in pUC9 could also only partially protect against the lethal stage of disease and none of those hamsters were protected against diarrhea. Lyerly et al. [(1990) Curr. Microbiol. 21:29] stated that antibodies to the C. difficile toxin A recombinant protein tested did not prevent the diarrheal stage of the disease and the death in half of the hamsters was due to the varying levels of neutralizing serum antibodies to the toxin A recombinant. From the above results, differences in anti-recombinant C. difficile toxin A titers seen between hamsters in a group may not explain why protection did not occur in all of the animals. The above results indicate that possibly an additional component, possibly a toxin B recombinant protein, is necessary for a more effective vaccine against C. difficile disease.

Detailed Description Text (918):
The recombinant proteins used for vaccination were the C. difficile toxin A recombinant protein pPA1870-2680N/C (Examples 11 and 29) and the C. difficile toxin B recombinant protein pPB1750-2360 (Example 15b). The recombinant proteins were expressed in the pET vector instead of pMal-c vector used in Example 38 because the proteins expressed and isolated using the pET vector were found to be capable of purification at a higher level of purity with lower levels of endotoxin. Production of recombinant proteins in the pET vector is especially amenable for the potential utilization of the recombinant protein as a human vaccine which demands high purity and low levels of potentially harmful endotoxin.

Detailed Description Text (928):
As shown in Table 47, one to three days after challenge with C. difficile, all of the hamsters immunized with either pPA1870-2680 or pB1750-2360 and the BSA control group developed diarrhea. All the hamsters in those three groups except two members immunized with pPA1870-2680, died from several hours to 48 hours after the detected onset of

diarrhea. Necropsy revealed severe enterocolitis in the animals with inflamed and enlarged cecums characteristic of C. difficile disease. In contrast, hamsters immunized with the vaccine comprising the combination of pPA1870-2680 or pB1750-2360 proteins showed no signs of illness such as diarrhea and remained healthy for the entire 14-day post-infection observation period. In fact, these animals have remained healthy for a period of at least 5 months post-infection; these results demonstrate that vaccination with the combination of pPA1870-2680 or pB1750-2360 proteins confers complete and long term protection on hamsters inoculated with C. difficile.

Detailed Description Text (929):
The protective effect seen with the combination vaccine was not due to differences in antibody titer in this group compared to the antibody titers in the hamsters vaccinated with only recombinant C. difficile toxin A or C. difficile toxin B. Protection of the hamsters immunized with the C. difficile toxin A/B combination (i.e., pPA1870-2680 and pB1750-2360) was statistically significant compared to the control; the P value was determined to be less than 0.001.

Detailed Description Text (930):
The above results demonstrate that recombinant C. difficile toxin A and toxin B proteins are both key components for an effective vaccine against C. difficile and that ellictation of antibodies against recombinant C. difficile toxins A or B alone was not sufficient to confer complete protection. Antibodies generated against a recombinant C. difficile toxin B in addition to recombinant C. difficile toxin A both confer protection and they both act synergistically to neutralize C. difficile-associated diarrhea and death. While the invention is not limited by any particular mechanism, the protection from the anti-C. difficile toxin serum antibodies may result from the leakage of the C. difficile toxin A and B neutralizing antibodies into tissues or the intestinal lumen during the inflammation that accompanies the early stages of C. difficile enterocolitis.

Detailed Description Text (952):
The immune IgY mixture was prepared by mixing an equal volume of an 8.times. concentrate of IgY raised against pMA1870-2680 and an equal volume of an 8.times. concentrate of IgY raised against pPB1750-2360; the resulting mixture was designated A-6/B-3 IgY. The amount of anti-toxin protein specific antibodies contained in this A-6/B-3 IgY mixture was about 1.2 mg/ml of anti-recombinant toxin A IgY and about 400 .mu.g/ml of anti-recombinant toxin B IgY. These amounts were determined by affinity purification as previously described in Example 15c. The amounts of total IgY in the 2 ml IgY dose was about 80 mg, and about 40 mg in the 1 ml dose.

Detailed Description Text (968):
Quantitation of specific IgY levels in the cecal samples was determined by comparing the antibody reactivity directed against either recombinant toxin A or recombinant toxin B to the reactivity generated using known amounts of affinity-purified IgY in ELISA assays. IgY specific for recombinant C. difficile toxins A and B were affinity purified as described in Example 15c. Briefly, affinity-purified antibodies were isolated from PEG-purified IgYs from the eggs of hens immunized with either pMA1870-2680 (Interval A-6) or pPB1750-2360 (Interval B-3). The PEG-purified anti-pMA1870-2680 IgY was affinity-purified using a column comprising pMA1870-2680 bound to Actigel (Sterogene). The PEG-purified anti-pPB1750-2360 IgY was affinity purified using an Actigel column containing pPB1850-2360 (Example 15c), a recombinant C. difficile toxin B protein that is 100 amino acids smaller than pPB1750-2360.

Detailed Description Text (969):
The affinity-purified anti-pMA1870-2680 IgY (A-6) and the affinity-purified anti-pPB1750-2360 IgY (B-3) were quantitated by measuring the absorbance at 280 nm and each preparation was diluted to a concentration of 10 .mu.g/ml in PBS. The affinity-purified IgYs were tested by ELISA starting with an initial concentration of 10 .mu.g/ml and at serial 5-fold dilutions in the respective coated-microtiter plate along side the cecal extracts. Rabbit anti-chicken IgY conjugated with alkaline phosphatase (Sigma) was used as the secondary antibody at a dilution of 1:750 to detect the bound IgYs in the ELISA.

Detailed Description Text (970):
Comparison of the ELISA reactivity equivalence between the cecal extracts to the known concentrations of the affinity-purified IgY allowed the amount of specific anti-recombinant IgY present in the cecal extracts to be estimated. From the ELISA results the amount of specific anti-pMA1870-2680 IgY (A-6) was found to be about 12 .mu.g/ml. The amount of specific anti-pPB1750-2360 IgY (B-3) was determined to be about

800 ng/ml. These concentrations of specific IgYs provide estimates of the effective therapeutic concentrations necessary to achieve protection at the site of infection. Since the amount of specific IgY given orally prior to collection of the cecal fluid was about 2400-2800 .mu.gs against the toxin A recombinant and from 400-800 .mu.gs against the toxin B recombinant, there was approximately a 200-233 fold reduction and 500-1000 fold reduction in the detectable amount of anti-recombinant toxin protein IgY found in the cecum directed against Interval A-6 and Interval B-3, respectively.

Detailed Description Text (975):
A sandwich ELISA assay was used to capture IgY in the cecal material as follows. Rabbit anti-chicken IgG (Capped at 0.1 .mu.g/ml in PBS was used to coat a microtiter plate (100 .mu.l per well) overnight at 4.degree. C. Both of the cecal extracts were tested at an initial dilution of 1:500 and at serial 5-fold dilutions to a final dilution of 1:312,500. All sample dilutions were tested in duplicate. Affinity-purified antibodies directed against recombinant toxin A (pPA1870-2680, Interval A-6)were diluted to 0.1 .mu.g/ml and then further diluted serially by five-fold to a final concentration of 0.16 ng/ml, was also tested by ELISA for allow for quantitation by comparison. After incubation and washing, rabbit anti-chicken alkaline phosphatase IgG (Sigma) was added (at 1:1000 dilution) to the plates. The plates were then washed and substrate (p-nitrophenyl phosphate) was added and the plates were evaluated as described in Example 13c.

Detailed Description Text (976):
As described above in Example 43(b), the ELISA reactivity obtained using the affinity purified anti-recombinant toxin A IgY was matched to that ELISA activity generated in dilutions of cecal extract, to quantitate the amount of total IgY found in the cecum of the treated hamster.

Detailed Description Text (977):
From the results of the ELISA assay, the amount of total IgY in the cecum of the treated hamster was estimated to be 50 .mu.g/ml. Affinity purification studies showed that total IgY preparations comprised about 7% or 3.5 .mu.g/ml IgY specific for recombinant toxin A (anti-A-6 IgY) and about 1-2% or 500-1000 ng/ml IgY specific for anti-recombinant toxin B (anti-B-3 IgY). The concentrations of both of the specific IgYs detected here correlates fairly closely with the amounts detected above in Example 43(b), namely, 3.5 .mu.g/ml versus 12 .mu.g/ml for anti-Interval A-6 and 800 ng/ml versus 500-1000 ng/ml for anti-Interval B-3.

Detailed Description Text (997):
The anti-pPA1870-2680 IgYs were tested in the hamster model along with antibodies raised against the toxin B protein also expressed using the pET vector (pPB1750-2360, Interval B-3). The use of a common expression system to produce both recombinant toxins has definite manufacturing advantages. For example, the same affinity-purification columns and protocols can be used for both recombinants and both antigens should be of comparable purity and yield.

Detailed Description Text (1001):
Hens were immunized with recombinant proteins expressed using the pET vector; nickel column affinity-purified recombinant toxin A (pPA1870-2680) or the recombinant toxin B (pPB1750-2360) proteins were mixed with either the Quil A (Accurate Scientific) or Gerbu (CC Biotech) adjuvants. These two adjuvants were chosen on the basis of performance (shown in Example 35) and cost. The immunization protocol followed was basically that described in Example 35.

Detailed Description Text (1003):
The IgYs were resuspended in 0.1M carbonate buffer, pH 9.5 at 8.times. yolk concentration (about 40 mg/ml) and an ELISA was performed (as described in Example 35) to determine the anti-recombinant toxin A and anti-recombinant toxin B titers. The antibody titers generated against either the pPA1870-2680 (A-6) or pPB1750-2360 (B-3) proteins using either the Gerbu or Quil A adjuvants was found to be 1:62,500. By affinity purification, the amount of specific A-6 and B-3 IgY using Gerbu was 4.3% and 1.0% respectively. The amount of specific IgY using Quil A was 2.2% for A-6 and 1.9% for B-3.

Detailed Description Text (1020):
Affinity Purification of Native C. difficile Toxin A Using Anti-Recombinant C. difficile Toxin A Antibodies

Detailed Description Text (1021):

Avian antibodies (IgY) raised against recombinant C. difficile toxin A protein were affinity purified using interval A-6 as the affinity ligand. The resulting specific antibodies were then immobilized on a solid support to purify native toxin A from C. difficile (ATCC #43255) organisms grown in dialysis bags submerged in BHI broth. The following example describes the a) affinity purification of avian antibodies directed against a recombinant fragment of toxin A and generation of a toxin A affinity column, b) growth of C. difficile organisms to produce toxin A and B in dialysis bag culture supernatants, c) affinity purification of toxin A, d) in vitro characterization of affinity purified C. difficile toxin A, e) investigation of an alternate strategy for coupling the anti-A-6 IgY to a solid support to affinity purify toxin A, f) affinity purification of C. difficile toxin A on affinity column generated by periodate oxidation of A-6 IgY and g) in vitro characterization of affinity purified C. difficile toxin A.

Detailed Description Text (1022):
a) Affinity Purification of Avian Antibodies Directed Against a Recombinant Fragment of Toxin A and Generation of a Toxin A Affinity Column

Detailed Description Text (1023):
Antibodies specific for Interval A-6 (aa 1870-2680) of C. difficile toxin A were affinity purified to provide reagents for the generation of an affinity column to permit purification of C. difficile toxin A from liquid culture supernatants and to provide an immunoassay reagent to permit detection of C. difficile toxin A in culture supernatant and affinity purified C. difficile toxin A samples.

Detailed Description Text (1024):
i) Affinity purification of A-6 IgY

Detailed Description Text (1025):
Hyperimmune IgY from eggs containing antibodies to A-6 recombinant protein using Freund's adjuvant was extracted using the PEG fraction method (Example 1). The antibody-containing supernatant was applied to a A-6 affinity column, made by covalently coupling pPA1870-2680 protein (prepared in Example 29) to Actigel A affinity resin (Sterogene Biochemicals) according to manufacturer's instructions. Approximately 10.2 mg of pPA1870-268 (A-6) protein was coupled to 5 ml Actigel affinity resin. The anti-A-6 IgY was eluted with Actisep elution media (Sterogene Biochemicals) as described in Example 15c, and dialyzed against PBS for 24-48 hours at 2.degree.-8.degree. C.

Detailed Description Text (1026):
ii) Coupling of Affinity-Purified Anti-A-6 IgY to an Activated Affinity Resin to Make a C. difficile Toxin A Affinity Column

Detailed Description Text (1027):
An initial toxin A affinity column was prepared as described in Example 48a below, by coupling the anti-A-6 IgY to Actigel A affinity resin. By comparing the pre- and post-coupling absorbance values of the IgY at 280 nm, it was estimated that 58%, or about 7.6 mg, of the anti-A-6 IgY was coupled to the affinity resin.

Detailed Description Text (1030):
The dialysis bag culture supernatant samples and a known toxin A sample purchased commercially were analyzed as described in Example 49b, section iv, with the exception that affinity purified A-6 IgY was used as the primary antibody for the western blot.

Detailed Description Text (1032):
Both culture supernatant samples analyzed appeared to contain immunoreactive C. difficile toxin A when analyzed by Western blot. This protein co-migrated with the commercial toxin A and was recognized by the affinity-purified anti-A-6 IgY. The culture supernatant samples were pooled prior to affinity purification of toxin A. The pooled culture supernatants were not concentrated prior to loading on the affinity column.

Detailed Description Text (1033):
c) Affinity Purification of C. difficile Toxin A

Detailed Description Text (1034):
The C. difficile toxin culture supernatant samples were affinity purified as described in Example 48c. The volume of the Actisep fraction following elution and dialysis was 42 ml, 15 ml of which were removed and concentrated to 3 ml prior to analysis. A

Centricon 30 concentrator (Amicon) was used to concentrate the sample.

Detailed Description Text (1036):
In order to determine the presence or absence of toxin A in the Actisep eluted sample and effluent from the affinity column, these samples were analyzed by SDS-PAGE and Western blot along with the culture supernatant starting material. These analyses were performed as described in section b above to evaluate the relative amount of toxin A in the samples and the efficiency of the affinity purification.

Detailed Description Text (1037):
The resulting Western blot is shown in FIG. 50. In FIG. 50, lanes 1-3 were incubated with pre-immune IgY as the primary antibody and lanes 4-6 were incubated with anti-A-6 IgY as the primary antibody. Lanes 1 and 4 contain culture supernatant starting material; lanes 2 and 5 contain column flow-through and lanes 3 and 6 contain affinity purified toxin A.

Detailed Description Text (1038):
The results shown in FIG. 50 demonstrated that immunoreactive toxin A was detected in the culture supernatant starting material and the Actisep fraction. Furthermore, no toxin A was observed in the column effluent sample, indicating most of the toxin was bound by the affinity column. There appeared to be significantly more toxin A in the starting material than in the Actisep fraction. Since the column effluent apparently contains no toxin A, the difference in toxin A amounts between the starting material and Actisep fraction suggested a significant amount of the toxin was still bound to column, even after Actisep elution. One possible explanation for the inability of the Actisep to elute all of the toxin A is the tendency for toxin A to bind nonspecifically to the carbohydrate region of molecules such as immunoglobulins. This is possible because the anti-A-6 IgY on the column is coupled via primary amines, which would allow for a subpopulation of the IgY to couple via the Fab region, leaving the carbohydrate-containing Fc region accessible for binding to toxin A.

Detailed Description Text (1039):
e) Investigation of an Alternate Strategy for Coupling the Anti-A-6 IgY to a Solid Support to Affinity Purify Toxin A

Detailed Description Text (1046):
The anti-A-6 IgY-Affi-Gel affinity resin was poured into a BioRad Econo column and the unbound antibody was washed through the resin and saved for A.sub.280 analysis. The resin was then washed with 1 bed volume of PBS (10 mM sodium phosphate, 0.5M NaCl, pH 7.2). This wash was also collected and saved for A.sub.280 analysis. The resin was then washed with several more volumes of PBS, and treated with the Actisep elution buffer (Sterogene Bioseparations) to ensure no unbound antibody remained in the resin. By comparing the pre-and post-coupling A.sub.280 values of the A-6 IgY, it was estimated that 95%, or 8.4 mg, of the IgY was coupled to the resin.

Detailed Description Text (1047):
f) Affinity Purification of C. difficile toxin A on Affinity Column Generated by Periodate Oxidation of Anti-A-6 IgY

Detailed Description Text (1048):
Two dialysis bag culture supernatants, grown as described in Example 48b, sections iv and v, were pooled and concentrated to about 10.5 ml using an Amicon centriprep concentrator. The pooled, concentrated supernatants were then applied to the anti-A-6 IgY Affi Gel affinity column and the column effluent was collected and reloaded several times to bind as much toxin as possible. The unbound protein was then removed by washing the column with several bed volumes of PBS and the bound toxin A was eluted with 2 bed volumes of Actisep elution media. The column effluent was saved for analysis to evaluate the efficiency of the affinity purification. The Actisep-eluted toxin was then dialyzed against TBS for 24-48 hours at 2.degree.-8.degree. C., and concentrated from 53 to 3 ml using a Centriprep concentrator (Amicon).

Detailed Description Text (1049):
g) In Vitro Characterization of Affinity Purified C. difficile Toxin A

Detailed Description Text (1053):
HPLC analysis was used to compare both the purity and retention times of the affinity purified toxin A samples. Commercial and affinity purified toxin A samples were applied to a Shodex KW 803 HPLC column and eluted with PBS, using a Waters HPLC system. The toxin A retention times were approximately 7 minutes for both toxin samples, suggesting

the toxins are identical. Furthermore, the purities of both toxins were similar.

Detailed Description Text (1054):
iii) Western Blot Analysis of Culture Supernatant Starting Material, Affinity Purified Toxin A and Column Effluent (flow through)

Detailed Description Text (1055):
In order to evaluate the efficiency of the affinity purification and immunochemically identify the affinity purified toxin A, the culture supernatant, affinity purified toxin A, and column effluent samples were electrophoresed by SDS-PAGE on a 5% gel under reducing conditions and transferred to nitrocellulose using standard methods. The blot was temporarily stained with 10% Ponceau S to allow the lanes to be marked and the remaining protein binding sites were blocked overnight at 2.degree.-8.degree. C. with a PBS solution containing 1 mg/ml dry milk. The blot was cut into two halves, one of which was incubated with anti-A-6 IgY primary antibody, diluted to 1 .mu.g/ml in PBS containing 1 mg/ml BSA, and the second half incubated with preimmune IgY diluted to 1 .mu.g/ml in PBS/BSA. After a two hour incubation in the presence of the primary antibody (with gentle agitation), the unbound primary antibody was removed with successive washes of PBS, BBS-Tween and PBS. Rabbit anti-chicken IgY alkaline phosphatase conjugated secondary antibody, diluted 1:2000 in PBS containing 1 mg/ml BSA was then added to each blot. After two hours, the blots were washed to remove unbound secondary antibody and developed with BLIP/NBT (Kirkegaard and Perry) substrate solution. Color development was stopped by flooding the blots with water. The resulting Western blot is shown in FIG. 51.

Detailed Description Text (1056):
In FIG. 51, lanes 1-7 were incubated with anti-A-6 IgY as the primary antibody and lanes 8-15 were incubated with pre-immune IgY as the primary antibody. Lanes 1 and 9 contain broad range molecular weight markers (BioRad). Lanes 2 and 10 contain C. difficile culture supernatant #1. Lanes 3 and 11 contain C. difficile culture supernatant #2. Lanes 4 and 12 contain C. difficile culture supernatants #1 and #2 (pooled). Lanes 5 and 13 contain column flow-through. Lanes 6 and 14 contain affinity purified Toxin A (high load; i.e., 2.times. the load shown in lanes 7 and 15). Lanes 7 and 15 contain affinity purified Toxin A (low load). Lane 8 does not contain any sample material (blank).

Detailed Description Text (1057):
The affinity purified toxin A sample (lane 7) was 3.5 fold more concentrated than the pooled starting material sample (lane 4); however, 1/3 the volume (5 .mu.l vs 15 .mu.l) of the affinity purified sample was loaded compared to the pooled starting material sample. Consequently, if most of the toxin A was recovered from the column, the toxin A levels detected on the Western blot should be similar. As shown in FIG. 51, the signals corresponding to the main high molecular weight bands are comparable. Therefore, the recovery of toxin A from the affinity column appeared to be quantitative.

Detailed Description Text (1059):
Affinity Purification of Native C. difficile Toxin B Using Anti-Recombinant C. difficile Toxin B Antibodies

Detailed Description Text (1060):
Arian antibodies (IgY) raised against recombinant C. difficile toxin B protein (pPB 1750-2360; Interval B-3) were affinity purified using Interval B-3 (i.e., aa 1750-2360 of C. difficile toxin B) as the affinity ligand. The resulting purified anti-Interval B-3 specific antibodies were then immobilized on a solid support to facilitate purification of native toxin B derived from C. difficile organisms (ATCC #43255) grown under conditions favorable for toxin production.

Detailed Description Text (1061):
The example involved a) affinity purification of avian antibodies directed against a recombinant fragment of C. difficile toxin B and generation of a C. difficile toxin B affinity column, b) growth of C. difficile organisms to produce toxins A and B in liquid culture and dialysis bag culture supernatants, c) affinity purification of C. difficile toxin B, and d) in vitro and in vivo characterization of affinity purified toxin B from C. difficile.

Detailed Description Text (1062):
a) Affinity Purification of Avian Antibodies Directed Against a Recombinant Fragment of C. difficile Toxin B and Generation of a C. difficile Toxin B Affinity Column

Detailed Description Text (1063):
Antibodies specific for Interval B-3 of C. difficile toxin B protein were affinity purified to provide reagents for the generation of an affinity column to permit purification of C. difficile toxin B from liquid culture supernatants and to provide an immunoassay reagent to permit detection of C. difficile toxin B in culture supernatants and affinity-purified C. difficile toxin B samples.

Detailed Description Text (1064):
i) Affinity Purification of Anti-Interval B-3 IgY

Detailed Description Text (1065):
Hyperimmune IgY was extracted from eggs containing antibodies to the Interval B-3 recombinant protein (pPB 1750-2360) generated using Gerbu adjuvant (see Example 45) using the PEG fractionation method (Example 1). The antibody-containing supernatant was applied to an Interval B-3 affinity column, made by covalently coupling pPB 1750-2360 protein (prepared in Example 29) to Actigel A affinity resin (Sterogene) as described in Example 15c. This fragment was chosen because it contains the C. difficile toxin B repeat region and does not contain regions of homology with the C. difficile toxin A protein, therefore the resulting purified antibody should not cross-react with C. difficile toxin A. The anti-Interval B-3 antibodies (anti-B-3 IgY) were eluted from the column with 4M guanidine HCl, pH 8.0 and dialyzed against PBS for 24 to 48 hours at 2.degree.-8.degree. C.

Detailed Description Text (1066):
ii) Coupling of Affinity-Purified Anti-B-3 IgY to an Activated Affinity Resin to Make a C. difficile Toxin B Affinity Column.

Detailed Description Text (1067):
A C. difficile toxin B affinity column was made by coupling 11 mg of the affinity purified avian anti-B-3 antibodies prepared above to 5 ml of Actigel affinity resin (Sterogene). A coupling time of 30 minutes was used rather than the minimum 2 hours recommended by the manufacturer in order to minimize the number of sites where each antibody molecule is coupled to the resin, thereby making the antibody more accessible to the toxin. In addition, the column was only exposed to high salt buffers or the Actisep elution buffer (Sterogene); no guanidine solutions were utilized during the preparation of the affinity column in order to minimize denaturation of the anti-B-3 antibodies. Comparison of the pre- and post-coupling absorbance values of the IgY at 289 nm, revealed that approximately estimate 62%, or about 6.8 mg, of the anti-B-3 IgY was coupled to the resin.

Detailed Description Text (1074):
In order to determine whether C. difficile toxin B was present in the culture supernatant, the supernatant was analyzed by native PAGE and Western blotting as follows. The harvested culture supernatant was concentrated about 10-fold using a Centricon 30 concentrator (Amicon) prior to electrophoresis on native PAGE gels. The concentrated sample was then mixed with an equal volume of native gel sample buffer (50% sucrose, 0.1% bromophenol blue) and loaded on a 4-15% Tris-glycine gradient gel (Bio-Rad), along with a known sample of C. difficile toxin B, purchased from Techlab. The samples were electrophoresed for 3 hours at 150 volts, constant voltage, using a Hoefer power supply. Following electrophoresis, the gel was cut in half and one half was stained with Coomassie blue and destained with a solution comprising 10% glacial acetic acid/40% methanol to visualize any protein bands. The other half of the gel was blotted and probed using affinity purified anti-B-3 antibody (section i above).

Detailed Description Text (1076):
As shown in FIG. 52, the commercial toxin B was detectable on the Coomassie stained gel. The concentrated culture supernatant showed several relatively faint bands, however no detectable proteins in the supernatant samples co-migrated with the known C. difficile toxin B sample. Furthermore, western blot analysis did not detect any proteins in the culture supernatant that were recognized by the affinity purified anti-B-3 antibody. These results demonstrated that the above growth conditions did not appear to be optimal for toxin production.

Detailed Description Text (1086):
A shown in FIG. 53, the presence of C. difficile toxin B was detected by incubating the blot strips with affinity purified anti-B-3 IgY. After washing the blots to remove unbound anti-B-3 antibodies, bound anti-B-3 antibodies were detected by incubating the strips with a secondary antibody comprising rabbit anti-chicken Ig conjugated to alkaline phosphatase (Sigma). The blots were washed again to remove any unbound

secondary antibody and the blots were developed in freshly prepared BLIP/NBT substrate solution. Development was stopped by flooding the blots with water once an adequate signal was obtained.

Detailed Description Text (1088):
Comparison of the commercial toxin B sample to the toxin B produced in the dialysis bag culture supernatant samples revealed that the culture supernatant sample contained a higher percentage of intact toxin B protein (i.e., there was much less evidence of degradation in the form of minor immunoreactive bands present in the culture supernatant samples). Because both culture supernatant samples contained toxin B (although at different concentrations), they were pooled prior to affinity purification.

Detailed Description Text (1089):
c) Affinity Purification of C. difficile Toxin B

Detailed Description Text (1090):
The dialysis bag culture supernatant samples were pooled and applied to the toxin B affinity column [prepared in section a)]. Nonspecific proteins were removed by washing the column with PBS until the baseline OD was achieved. The bound protein was eluted using Actisep elution media (Sterogene) and was then dialyzed against Tris-buffered saline, pH 7.5 (50 mM Tris, 150 mM NaCl). Following dialysis, the affinity purified protein was concentrated from 40 ml to 4.5 ml using a Centricon 30 concentrator (Amicon).

Detailed Description Text (1091):
d) In Vitro and In Vivo Characterization of Affinity Purified Toxin B From C. difficile

Detailed Description Text (1092):
In order to determine the presence or absence of C. difficile toxin B in the Actisep-eluted sample and effluent from the affinity column (i.e., the flow-through), these samples were analyzed by native PAGE and Western blotting along with the culture supernatant starting material. These analyses were performed to evaluate the relative amount of C. difficile toxin B in the culture supernatant and the efficiency of the affinity purification.

Detailed Description Text (1093):
The affinity purified, culture supernatant, flow-through, and commercial C. difficile toxin B samples were each mixed with an equal volume native sample buffer and loaded on a 4-15% native Tris-glycine gradient gel (Bio-Rad). The sample were electrophoresed for approximately 2.5 hours at 200 volts, constant voltage, using a Hoefer power supply, and transferred to nitrocellulose using a semi-dry blotting apparatus (Millipore) according to manufacturer's instructions. The blot was blocked overnight using a solution containing 1% powdered milk in PBS. The blot was then incubated with affinity purified anti-B-3 IgY as the primary antibody and rabbit anti-chicken conjugated to alkaline phosphatase as the secondary antibody. The blots were handled as described in section b(vi) to permit visualization of the C. difficile toxin B protein.

Detailed Description Text (1094):
FIG. 54 shows the Coomassie stained gel and corresponding Western blots. In FIG. 54, lanes 1-3 were stained with Coomassie blue; lanes 5-10 were probed with anti-B-3 IgY and lanes 8-10 were probed with pre-immune IgY. Lanes 1, 5 and 8 contain affinity purified toxin B; lanes 2, 6 and 9 contain the column flow through; lanes 3, 7 and 10 contain commercial toxin B (Techlab). Lane 4 does not contain any protein (blank).

Detailed Description Text (1095):
The following results were obtained upon western blot analysis. All three samples (culture supernatant, eluted protein and flow-through) contained immunoreactive toxin B. These results indicated that the affinity purification protocol was successful in purifying some toxin B. However, as the flow-through fraction was found to contain significant amounts of toxin B the following modifications would be examined for the ability to further optimize the purification process (e.g., coupling of B-3 IgY to Affigel hydrazide support (BioRad) via periodate oxidation of IgY).

Detailed Description Text (1096):
ii) Yield of Affinity Purified C. difficile Toxin B

Detailed Description Text (1097):

The yield of affinity purified C. difficile toxin B was determined by BCA protein assay (Pierce), using BSA as the protein standard. This assay showed that the toxin B concentration was 73 .mu.g/ml.times.4.5 ml (volume of affinity purified material)=365 .mu.g of toxin B. Approximately 70 ml of dialysis bag culture supernatant was used as the starting material; therefore, about 5 .mu.g toxin B was recovered per milliliter of culture. This yield was consistent with previously reported yields using this method of culturing C. difficile [7.8 .mu.g toxin B/ml of culture supernatant; Meador and Tweten (1988), supra].

Detailed Description Text (1098):
iii) Measurement of the In Vivo Activity of the Affinity Purified C. difficile Toxin B

Detailed Description Text (1099):
The in vivo activity of the affinity purified C. difficile toxin B was determined by injecting various amounts of the purified toxin B preparation (described below) into 30 to 40 gram female syrian hamsters. Another group of hamsters was injected with various amounts of a commercial toxin B preparation (TechLabs) for comparison with results previously obtained. The LD.sub.100 of the TechLabs preparation of C. difficile toxin B was found to be about 5 .mu.g for 30-40 g hamsters when administered I.P. (Example 19). At this concentration (5 .mu.g/30-40 g hamster), the hamsters died within about 3 hours post-I.P. injection.

Detailed Description Text (1100):
The LD.sub.100 concentration of the affinity purified toxin B was determined by I.P. injection of 1 ml of a solution containing either 5 or 50 .mu.g of affinity purified toxin B diluted in saline. Two 30-40 gram hamsters were injected with each concentration of affinity purified toxin B. The hamsters injected with 50 .mu.g of affinity purified material hamster died within 2 hours; the hamsters injected with 5 .mu.g of affinity purified toxin B died within 4 hours. These results demonstrated that the toxicity of the affinity purified C. difficile toxin B preparation was comparable to the commercially available C. difficile toxin B.

Detailed Description Text (1103):
In this example, a rapid agglutination assay designed to detect C. difficile toxin A and toxin B in either culture supernatants or biological specimens such as feces was developed. Affinity purified antibodies against recombinant C. difficile toxin A and toxin B from hens were used to passively coat small polystyrene particles. In principle, the particles coated with the specific avian antibodies (IgY) to toxin A and toxin B should form visible aggregates when they are mixed with a sample containing the toxins. This format should produce a specific, sensitive and rapid assay. Affinity purified IgY in this case confers specificity and sensitivity to C. difficile toxin, while ease of use and speed of the assay is conferred using an agglutination assay format. This example describes: a) initial development of the agglutination assay for the detection of C. difficile toxin A and toxin B; and b) evaluation and optimization of the agglutination assay.

Detailed Description Text (1105):
Antibodies were generated in hens using the toxin A recombinant (pMAL 1870-2680) and the toxin B recombinant (pPB1750-2360) using Freund's adjuvant as described in previous Examples. The recombinant toxin A antibodies (A-6 IgY) and the recombinant toxin B antibodies(B-3 IgY) were PEG fractionated the then affinity purified as described in Example 15c. The A-6 IgY was affinity purified against pPA1870-2680 and the B-3 IgY was affinity purified against pB1750-2369. The affinity-purified antibodies were then passively coated onto the polystyrene particles.

Detailed Description Text (1106):
For each IgY preparation to be coated, 100 .mu.l of a 5% bead suspension of 1.mu. beads (Spherotech Inc., Libertyville, Ill.) was removed and centrifuged for 2 minutes at 14,000.times.g in a Beckman microfuge to pellet the particles. The particles were then washed with TBS (10 mM Tris, 150 mM NaCl, pH 8) PBS-Tween (10 mM sodium phosphate, 150 mM NaCl, pH 7.2+0.05% Tween 20) and TBS. The particles were centrifuged for 2 minutes following each wash and the wash buffer was discarded. Following the last TBS wash, the particles were resuspended in 1 ml of the antibody coating solution; affinity purified avian A-6 or B-3 IgY at 100 .mu.g/ml in TBS. PEG-fractionated preimmune IgY was also coated in the same manner to serve as a negative control in the agglutination assays. The particle suspensions were then inverted at room temperature for 18 to 24 hours to allow the IgY to coat the particles.

Detailed Description Text (1109):

In order to demonstrate the feasibility of using affinity purified polyclonal avian IgY in this type of assay, the ability of A-6 IgY coated particles to agglutinate in the presence of various concentrations of toxin A was evaluated.

Detailed Description Text (1112):
Affinity purified polyclonal avian antibodies were most commonly used to coat the particles, however the use of PEG-fractionated and water-diluted IgY preparations was also investigated, in order to determine if it was possible to increase the sensitivity of the agglutination assay by using polyclonal antibodies which might contain a population of high affinity antibodies lost during affinity purification.

Detailed Description Text (1113):
PEG-fractionated polyclonal A2 IgY was used to coat 1.mu. polystyrene particles under conditions identical to those described above, and the particles were evaluated for sensitivity in the C. difficile toxin A agglutination assay. These particles were less sensitive than particles coated with affinity purified IgY.

Detailed Description Text (1115):
It was determined that particles coated with water-diluted IgY preparations were less sensitive than particles coated with affinity purified IgY. Affinity purified IgY therefore appears superior to batch-fractionated IgY preparations in this assay format. In order to increase the sensitivity and maintain the specificity of the agglutination assays, we then evaluated the effect of several other variables on the assay performance.

Detailed Description Text (1117):
A-6 and B-3 IgY-coated beads were evaluated for their agglutinability with lowest amount of toxin (i.e., sensitivity) and specificity. Instead of using PEG-fractionated preimmune IgY, affinity-purified IgY against an irrelevant antigen, C. atrox snake venom, was used to coat the particles as a negative control. Toxin A and toxin B were serially diluted in PBS from 1 .mu.g/ml to 0.1 ng/ml. Ten .mu.l of bead suspension was mixed with 20 .mu.l sample in wells of glass agglutination plates, mixed well and rotated on nutator (Lab Quake) or manually for two minutes. Agglutination was read after two minutes. A completely uniform suspension was rated as "-," a slightly gritty appearance was rated as ".+-." and distinct agglutination was rated as "+" or "++," according to the size of the aggregates.

Detailed Description Text (1119):
The density of the beads in final suspension was also evaluated. In order to improve the sensitivity and specificity, A-6 or B-3 IgY-coated latex particles were tested for their agglutinability at 2.5%, 1.25%, 0.5%, and 0.25% suspensions. All the bead suspensions except 2.5% resulted in no or low signal. Antibodies generated using different adjuvants have different avidities and affinities, and hence agglutinate differently. It was known that antibodies with higher avidity and affinity form large and distinct aggregates. A-6 and B-3 IgY generated using Freund's and Gerbu as adjuvants were evaluated for their agglutinability at lowest concentration of toxin. Antibodies generated using Gerbu adjuvant were found to be better in giving distinct and large aggregates at 10-times lower concentrations of toxin A or toxin B, compared to antibodies generated with Freund's adjuvant.

Detailed Description Text (1123):
Five mg polystyrene particles (1.mu., Spherotech Inc., Libertyville, Ill.) were added to a tube and washed with 1 ml of TBS, PBS-T, and TBS followed by another wash with 50 mM Na.sub.2 CO.sub.3, pH 9.5 buffer. The beads were resuspended in the latter buffer to a total volume of 1 ml. A-6 IgY (affinity-purified, Gerbu-generated) was added to beads to a final concentration of 250 .mu.g/ml and incubated at room temperature on a nutator overnight. The next day, IgY-sensitized particles were washed with TBS, PBS-T, and TBS and resuspended in TBS to a final concentration of 2.5%. These IgY-sensitized particles were stored at 4.degree. C. until use.

Detailed Description Text (1125):
Five mg polystyrene particles (1.mu., Spherotech Inc., Libertyville, Ill.) were added to a tube and washed with 1 ml of TBS, PBS-T, and TBS followed by another wash with 50 mM Na.sub.2 CO.sub.3, pH 9.5 buffer. The beads were resuspended in the latter buffer to a total volume of 1 ml. B-3 IgY (affinity-purified, Gerbu-generated) was added to beads to a final concentration of 100 .mu.g/ml and incubated at room temperature on a nutator overnight. The next day, IgY-sensitized latex particles were washed with TBS, PBS-T, and TBS and resuspended in TBS to a final concentration of 2.5%. These IgY-sensitized latex particles were stored at 4.degree. C. until use.

Detailed Description Text (1155):
From the above it is clear that the present invention provides antitoxins and vaccines for the treatment and prevention of C. difficile disease. Furthermore, these antitoxins prevent the relapse of C. difficile disease which is commonly seen using conventional treatment protocols. Additionally, the invention provides a rapid agglutination assay for the detection of C. difficile toxins A and B in samples.

Detailed Description Paragraph Table (1):
TABLE 2 Clostridial Toxins Organism Toxins and Disease-Associated Antigens C. botulinum A, B, C.sub.1, C.sub.2, D, E, F, G C. butyricum Neuraminidase C. difficile A, B, Enterotoxin (not A nor B), Motility Altering Factor, Low Molecular Weight Toxin, Others C. perfringens .alpha., .beta., .epsilon., .iota., .gamma., .delta., .nu., .theta., .kappa., .lambda., .mu., .upsilon. C. sordelli/ HT, LT, .alpha., .beta., .gamma. C. bifermentans C. novyi .alpha., .beta., .gamma., .delta., .epsilon., .zeta., .nu., .theta. C. septicum .alpha., .beta., .gamma., .delta. C. histolyticum .alpha., .beta., .gamma., .delta., .epsilon. plus additional enzymes C. chauvoei .alpha., .beta., .gamma., .delta.

Detailed Description Paragraph Table (15):
TABLE 16
Purification Of Recombinant Toxin A Protein % Intact Yield Affinity Soluble Yield Intact Protein Purified Soluble Fusion Insoluble Clone.sup.(a) Solubuility Protein.sup.(b) Protein.sup.(c) Fusion Protein
pMA30-270 Soluble 4 mg/500 mls 10% NA PMA30-300 Soluble 4 mg/500 mls 5-10% NA pMA300-660 Insoluble -- NA 10 mg/500 ml pMA660- Soluble 4.5 mg/500 mls 50% NA 1100 pMA1100- Soluble 18 mg/500 mls 10% NA 1610 pMA1610- Both 22 mg/500 mls 90% 20 mg/500 ml 1870 pMA1450- Insoluble -- NA 0.2 mg/500 ml 1870 pPA1100- Soluble 0.1 mg/500 mls 90% NA 1450 pPA1100- Soluble 0.02 mg/500 90% NA 1870 mls pMA1870- Both 12 mg/500 mls 80% NA 2680 pPA1870- Insoluble -- NA 10 mg/500 ml 2680
.sup.(a) pP = pET23 vector, pM = pMALc vector, A = toxin A. .sup.(b) Based on 1.5 OD.sub.280 = 1 mg/ml (extinction coefficient of MBP). .sup.(c) Estimated by Coomassie staining of SDSPAGE gels.

Detailed Description Paragraph Table (22):
TABLE 23 Summary Of Toxin B Expression Constructs.sup.a Clone Affinity Tag Yield (mg/liter) % Full Length
pPB10-1750 none low (estimated from ? Western blot hyb.) pPB10-1530 none low (as above) ? pMB10-470 MBP 15 mg/l 0% pPB10-520 poly-his 0.5 mg/l 20% pPB10-330 poly-his >20 mg/l (insoluble) 90% pMB10-330 MBP 20 mg/l 10% pMB260-520 MBP 10 mg/l 50% pMB510-1110 MBP 25 mg/l 5% pMB510-820 MBP degraded (by Western blot hyb) pMB820-1110 MBP 20 mg/l 90% pMB1100-1750 MBP 15 mg/l 0% pMB1100-1530 MBP 40 mg/l 5% pMB1570-1750 MBP 3 mg/l <5% pPB1530-1750 poly-his no purified ? protein detected pMB1530-1750 MBP 20 mg/l 25% pMB1750-2360 MBP >20 mg/l >90% pMB1750-2360 MBP 6.5 mg/l (secreted) 50% pPB1750-2360 poly-his >20 mg/l >90% pMB1750-1970 MBP >20 mg/l >90% pMB1970-2360 MBP 40 mg/l >90% pMB1970-2360 MBP (no secretion) NA pMB1850-2360 MBP 20 mg/l >90% pPB1850-2360 poly-his 15 mg/l >90% pMB1850-1970 MBP 70 mg/l >90% pPB1850-1970 poly-his >10 mg/l (insoluble) >90% pPB1850-2070 poly-his >10 mg/l (insoluble) >90% pPB1750-1970(c) poly-his >10 mg/l (insoluble) >90% pPB1750-1970(n) poly-his >10 mg/l (insoluble) >90%
.sup.a Clones in italics are clones currently utilized to purify recombinant protein from each selected interval.

Detailed Description Paragraph Table (25):
TABLE 26 Neutralization Of Toxin B By Affinity Purified Antibodies Number Of Number Of Treatment Group.sup.a Animals Alive.sup.b Animals Dead.sup.b Preimmune.sup.1 0 5 CTB.sup.1 ; 400 .mu.g 5 0 CTB (AP on pPB1750-2360);.sup.2 875 .mu.g 5 0 CTB (AP on pMB1750-1970);.sup.2 875 .mu.g 5 0 CTB (AP on pMB1970-2360);.sup.2 500 .mu.g 5 0
.sup.a C. difficile toxin B (CTB) (Tech Lab; at 5 .mu.g/ml, 25 .mu.g total) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37.degree. C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either: .sup.1 4X antibody PEG prep or .sup.2 affinity purified (AP) antibody (from CTB PEG prep, on indicated columns). The amount of specific antibody in each prep is indicated; the amount is directly

determined for affinity purified preps and is estimated for the 4X CTB as described in Example 15. .sup.b The numbers in each group represent numbers of hamsters dead or alive, 2 hr post IP administration of toxin/antibody mixture.

Detailed Description Paragraph Table (27):
TABLE 28 In Vivo Neutralization Of Toxin B Using Affinity Purified Antibodies Number Animals Number Animals Treatment Group.sup.a Alive.sup.b Dead.sup.b Preimmune(1) 0 5 CTB(1) 5 0 pPB1750-2360(1) 5 0 1.5 mg anti-pMB1750-2360(2) 1 4 1.5 mg anti-pMB1970-2360(2) 0 5 300 .mu.g anti-CTB(2) 5 0 .sup.a C. difficile toxin B (CTB) (at 5 .mu.g/ml; 25 .mu.g total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37.degree. C. After incubation, 1 ml of this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either (1) 4X antibody PEG prep or (2) affinity purified antibody (on a pBP17502360 resin), either 1.5 mg/group (antipMB1750-2360 and antipMB1970-2360; used undiluted affinity purified antibody) or 350 .mu.g/group (antiCTB, repeat specific; used 1/5 diluted antiCTB antibody) .sup.b The numbers in each group represent numbers of hamsters dead or alive, 2 hr postIP administration of toxin/antibody mixture.

Detailed Description Paragraph Table (29):
TABLE 30 In Vivo Neutralization Of Toxin B Tested Antigen In vivo Immunogen Adjuvant Preparation.sup.a Utilized For AP Neutralization.sup.b Preimmune NA.sup.1 PEG NA no CTB (native) Titermax PEG NA yes CTB (native) Titermax AP pPB1750-2360 yes CTB (native) Titermax AP pPB1850-2360 yes CTB (native) Titermax AP pPB1750-1970 yes CTB (native) Titermax AP pPB1970-2360 yes pMB1750-2360 Freunds PEG NA no pMB1750-2360 Freunds AP pPB1750-2360 no pMB1750-2360 Gerbu PEG NA yes pMB1970-2360 Freunds PEG NA no pMB1970-2360 Freunds AP pPB1750-2360 no pPB1750-2360 Freunds PEG NA yes pPB1850-2360 Freunds PEG NA no pMB1850-2360 Freunds PEG NA no INT 1 + 2 Freunds PEG NA no INT 4 + 5 Freunds PEG NA no .sup.a Either PEG preparation (PEG) or affinity purified antibodies (AP). .sup.b `Yes` denotes complete neutralization (0/5 dead) while `no` denote no neutralization (5/5 dead) of toxin B, 2 hours postadministration of mixture. .sup.1 `NA` denotes not applicable.

Detailed Description Paragraph Table (30):
TABLE 31 In vivo Neutralization Of Toxin B Using Affinity Purified Antibodies Number Animals Number Animals Treatment Group.sup.a Alive.sup.b Dead.sup.b Preimmune.sup.i 0 5 CTB (300 .mu.g).sup.2 5 0 CTB (100 .mu.g).sup.2 1 4 pMB1750-2360 (G) (5 mg).sup.2 5 0 pMB1750-2360 (G) (1.5 mg).sup.2 5 0 pMB1750-2360 (G) (300 .mu.g).sup.2 5 0 pMB1750-2360 (F) (1.5 mg).sup.2 0 5 pPB1750-2360 (F) (1.5 mg).sup.2 5 0 pPB1750-2360 (F) (300 .mu.g).sup.2 1 4 CTB (100 .mu.g).sup.3 2 3 pPB1750-2360 (F) (500 .mu.g).sup.1 5 0 .sup.a C. difficile toxin B (CTB) (Tech Lab) at lethal concentration to hamsters (25 .mu.g) was added to the antibody (amount of specific antibod is indicated) and incubated for one hour at 37.degree. C.. After incubation, this mixture was injected IP into hamsters (1/5 total mix injected per hamster). Each treatment group received toxin premixed with antibody raised against the indicated protein (G = gerbu adjuvant, F = Freunds adjuvant). .sup.1 indicates the antibody was a 4X IgY PEG prep; .sup.2 indicates the antibody was affinity purified on a pPB18502360 resin; and .sup.3 indicates that the antibody was a 1X IgY PEG prep. .sup.b The numbers in each group represent numbers of hamsters dead or alive, 2 hrs post IP administration of toxin/antibody mixture.

Detailed Description Paragraph Table (33):
TABLE 34 C. difficile Toxin A Detection In Stool Using Affinity-Purified Antibodies Against Toxin A ng Toxin A/Welll OD.sub.410 Readout 200 0.9 100 0.8 50 0.73 25 0.71 12.5 0.59 6.25 0.421 0 0

Detailed Description Paragraph Table (34):
TABLE 35 C. difficile Toxin B Detection In Stool Using Affinity-Purified Antibodies Against Toxin B ng Toxin B/Well OD.sub.410 Readout 200 1.2 100 0.973 50 0.887 25 0.846 12.5 0.651 6.25 0.431 0 0.004

Detailed Description Paragraph Table (35):

TABLE 36 Yield Of Affinity Purified C. botulinum C Fragment/MBP Fusion Proteins Yield (mg/liter Percentage Of Total Construct of Culture) Soluble Protein pMABot 24 5.0 pMCABot 34 5.0 pMNABot 40 5.5 pMBot 22 5.0 pMA1870-2680 40 4.8

Detailed Description Paragraph Table (36):
TABLE 37
Determination Of Anti-C. botulinum Type A Toxin Serum IgG Titers Following Immunization With C. botulinum C Fragment-Containing Fusion Proteins Nasal Oral Route of Immunization pMBot & pMBot& PRE- pMA1870- pMA1870- Immunogen IMMUNE pMBot 2680 pMABot pMBot 2680 pMABot
Dilution 1:30 0.080 1.040 1.030 0.060 0.190 0.080 0.120 1:150 0.017 0.580 0.540 0.022 0.070 0.020 0.027 1:750 0.009 0.280 0.260 0.010 0.020 0.010 0.014 1:3750 0.007 0.084 0.090 0.009 0.009 0.010 0.007 # Rats 5 5 5 5 2 2 Tested
*Numbers represent the average values obtained from two ELISA plates, standardized utilizing the preimmune control.

Detailed Description Paragraph Table (37):
TABLE 38
Determination Of Anti-C. botulinum Type A Toxin Serum IgG Titers Following Immunization With C. botulinum C Fragment-Containing Fusion Proteins PRE- Immunogen IMMUNE pMBot pMABot pMNABot pMNABot
Dilution 1:30 0.040 0.557 0.010 0.015 0.010 1:150 0.009 0.383 0.001 0.003 0.002 1:750 0.001 0.140 0.000 0.000 0.000 1:3750 0.000 0.040 0.000 0.000 0.000 # Rats Tested 1 1 3 3

Detailed Description Paragraph Table (38):
TABLE 39 Determination Of The LD.sub.50 Of Purified C. botulinum Type A Toxin Complex Dilution Number Dead At 72 hr
1:320 2/2 1:640 2/2 1:1280 2/2 1:2560 0/2 (sick after 72 hr) 1:5120 0/2 (no symptoms)

Detailed Description Paragraph Table (40):
TABLE 40
Anti-C. botulinum Type A. Toxiod Serum IgG Titers In Individual Mice Immunized With pMBot or pHisBot Protein Preimmune.sup.1 pMBot.sup.2 pHisBot.sup.2 Sample Dilution Sample Dilution Sample Dilution 1:25 1:125 1:25 1:125 1:25 1:125 Mouse # 1:50 0 0 1:6250 1:50 0 0 1:6250 1:50 0 0 1:620
1 0.67 0.19 0.055 0.007 1.57 0.79 0.320 0.093 8 0 4 9 2 1.16 0.93 0.254 0.075 1.51 0.82 0.409 0.134 1 1 3 9 3 1.36 0.45 0.195 0.041 1.59 1.02 0.453 0.122 4 8 6 8 4 1.62 1.18 0.334 0.067 1.55 0.84 0.348 0.090 2 9 2 0 5 1.61 1.03 0.289 0.067 1.62 1.58 0.895 0.233 2 0 9 0 6 0.91 0.24 0.069 0.013 1.48 0.95 0.477 0.145 3 2 5 2 7 0.91 0.23 0.058 0.014 1.52 0.72 0.269 0.069 0 5 4 5 8 0.74 0.23 0.058 0.014 1.27 0.42 0.116 0.029 7 4 4 7 Mean 0.04 0.02 0.011 0.002 1.13 0.56 0.164 0.037 1.51 0.89 0.411 0.114 Titer 8 1 3 4 8 6
.sup.1 The preimmune sample represents the average from 2 sets of duplicate wells containing serum from a individual mouse immunized with recombinant Staphylococcus enterotoxin B (SEB) antigen. This antigen is immunologically unrelated to C. botulinum toxin and provides a control serum. .sup.2 Average of duplicate wells.

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